STUDIES ON THE ALLEGED TOXIC ACTION OF COD LIVER OIL

OBSERVATIONS ON GROWTH AND PATHOLOGIC CHANGES IN ANIMALS FED LARGE AMOUNTS OF COD LIVER OIL

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THREE FIGURES

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During the last 12 years there has appeared a long series of papers by Scandinavian workers describing toxic effects following the administration of cod liver oil (Slagsvold, '25; Wahlin, '31; Andersson, '33; Agduhr, '26, '36). Agduhr ('27) working with many species of animals (mice, rats, rabbits, calves, pigs and dogs), claims that pathologic changes may be produced by the oil even when given in daily doses as low as 0.1 cc. per kilogram body weight. Animals of different species were found to differ in their sensitivity to the toxic action of cod liver oil, rabbits and mice being much more susceptible than rats and dogs. The most serious and characteristic lesion was degeneration and fibrosis of the cardiac musculature, although sometimes lesions of the liver, adrenals, kidney and other organs were also noted (Agduhr, '29). In an extensive study of the electrocardiograms of animals in which lesions were produced by the feeding of cod liver oil, Agduhr and Stenström ('29) reported that the conducting tissue was functionally damaged. The toxic factor was found to be associated with the fatty acid fraction of the oil, although in a

[1] Standard Brands, Inc. Fellow, 1934–1936.

later investigation by Agduhr ('36) on the effect of components of cod liver oil upon the tissues of the mouse, lesions in the heart were also observed when the non-saponifiable fraction was fed.

Warning against the injudicious use of cod liver oil has come from a Stockholm clinic. Malmberg ('29) reported two cases of premature infants which were given cod liver oil and showed at autopsy heart lesions similar to those described by Agduhr. One child received 5 to 10 cc. of cod liver oil a day; the other was given 15 to 20 cc. of the oil daily. On the basis of the findings in these two infants, which lived 4 months and 16 days, respectively, and the autopsy reports of which included, in one case acute pneumonia and rickets (craniotabes and internal rachitic rosary), and in the other spina bifida cystica, paralysis of both lower limbs and meningitis, Malmberg states: "It seems that Agduhr's investigations as well as the findings in the cases submitted above . . . . should lead to much greater caution as regards dosage than hitherto has been the case . . . ."

In this country, Madsen, McCay and Maynard ('35) published a series of experiments which strongly suggested that there was a relationship between the ingestion of cod liver oil and skeletal muscle degeneration of Herbivora fed synthetic diets. The heart was also frequently involved. In a later publication Madsen ('36), although he confirmed the previous studies, showed further that cod liver oil was not the only factor concerned in the production of this dietary disease, and that much is left "to be explained as to the causes of the dystrophy which is produced in Herbivora by dietary means."

Cox and Roos ('34) were not able to demonstrate any lesions in rats fed for 130 days a diet in which cod liver oil furnished 78% of the calories. Agduhr's comments ('34) on the negative findings reported by Bell, Gregory and Drummond ('33) would also pertain here: namely, that in spite of the large amount of the oil fed, the experimental period was too short to produce the characteristic lesions in the rat, which at the outset is one of the species least susceptible to the 'toxic' action of cod liver oil.

Evidence has accumulated indicating that diets containing 10 to 15% cod liver oil produce retardation of growth of the albino rat, and that the addition of a liberal amount of yeast to such diets counteracts the unfavorable effect (Harris and Moore, '28; Bell, Gregory and Drummond, '33; De Vries and Puister, '33; Yamamoto, '34). Norris and Church ('30), in their assays of various fish liver oils, also noted this effect of yeast and thought that the toxicity was due to nitrogenous bases (isoamylamine and choline), but Bell and co-workers ('33) found no experimental basis for these conclusions. They demonstrated that the subnormal growth was not due to overdosage of vitamin A or vitamin D; they were unsuccessful in isolating the factor responsible for this effect.

Inasmuch as it is claimed that the mouse is highly susceptible to the toxic effects of cod liver oil (Agduhr and Stenström, '29), the present report deals in part with the effects of feeding large amounts of this oil to mice. In the investigation of the influence of yeast upon the growth of animals fed a high level of cod liver oil, rats were also used, and necropsy findings of those which lived over 150 days are included in the report on pathology.

EXPERIMENTAL PROCEDURE

Diets. The rats were taken at weaning and given a basal diet of constant composition with respect to percentage of foodstuffs; the variant was the type of fat used. The amount of yeast supplement differed among the groups, as discussed in the next section. The constituents of this basal ration were as follows: casein (washed with dilute acetic acid), 24%; rice starch, 45%; fat, 27%, and salt mixture (Osborne and Mendel), 4%. Diet I contained 27% of cod liver oil (Scott and Bowne) which furnished 47% of the total calories. Diet II contained 18% cod liver oil and 9% peanut oil; it furnished 32% of the total calories as cod liver oil. In diet III the source of fat was peanut oil; the ration was supplemented with 40 mg. weekly of a cod liver oil concentrate.[2]

[2] This concentrate was kindly furnished by the Health Products Corporation, Newark, New Jersey. The material had a potency of 72,000 U.S.P. (revised, 1934) units of vitamin A and 9,000 U.S.P. (revised, 1934) units of vitamin D.

The mice were weaned at 21 days of age and received thereafter diets containing 20% by weight of cod liver oil or peanut oil. The amount of yeast included in the rations, however, varied in the two major groups, one containing twice as much yeast as the other.

TABLE 1

Composition of diets for mice

CONSTITUENTS	DIET A	DIET B [1]
	%	%
Casein (86% protein)	25	20
Corn starch	39	34
Fat (cod liver oil or peanut oil)[2]	20	20
Yeast [3]	10	20
Osborne and Mendel salt mixture	5	5
Calcium carbonate [4]	1	1

[1] The total content of protein (which includes that derived from the yeast) is approximately the same in both diets.

[2] Mead Johnson cod liver oil was used and comprised 40% of the total calories. Diets containing peanut oil were supplemented with 40 mg. weekly of a cod liver oil concentrate already described (footnote 2 in text).

[3] Fleischmann yeast no. 2019, having a potency of approximately 15 to 20 Sherman units of vitamin B and 15 to 20 Sherman units of vitamin G.

[4] In order to correct for the large amount of phosphorus in the yeast, the diets were analyzed for calcium and phosphorus, and a calculated amount of calcium carbonate was added to bring the Ca/P ratio to 1.5.

Postmortem examinations and histologic technic. The mice were killed at intervals up to 300 days after the beginning of the experiment, and complete necropsies were performed. The organs of interest, which always included the heart, liver, right kidney and muscle of the right hind leg, were fixed in a solution of formaldehyde, U.S.P. (1:10). Blocks of these tissues were embedded in paraffin and the sections stained with hematoxylin-eosin.

Necropsies were also performed on those rats which were allowed to survive after the completion of the growth studies. The material routinely fixed for microscopic examination was the same as that for mice.

DISCUSSION AND RESULTS

Growth studies. Within each group of rats receiving one of the diets described in the preceding section the amount of yeast supplement varied. Thus, in the groups of rats fed diets I, II and III, respectively, eight of the sixteen animals in the group were given daily 400 mg. of yeast and the remaining eight were given 800 mg. of yeast. The composite growth curves for these groups are presented in figure 1 (B and C).

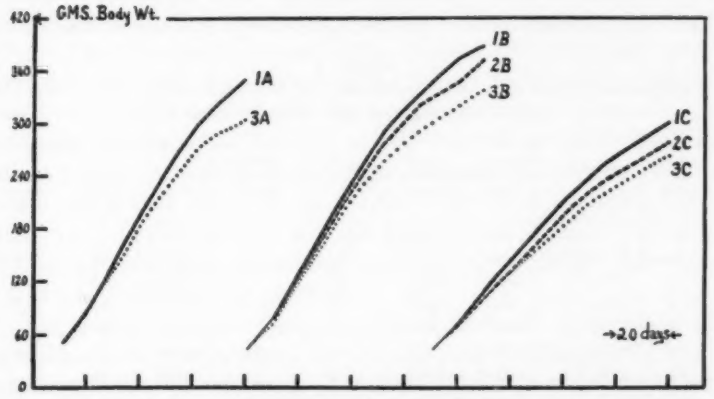

Fig. 1 Composite growth curves of male rats, showing that the differences in growth between animals receiving peanut oil and cod liver oil, respectively, are not affected by varying the amount of yeast supplement. The dotted lines (3A, 3B, 3C) represent the groups which were fed the ration containing 27% cod liver oil (diet I of text). The interrupted lines (2B, 2C) designate the growth of animals given the 18% cod liver oil diet (diet II). The continuous lines (1A, 1B, 1C) represent the control animals given the ration containing peanut oil (diet III). C, 400 mg. yeast. B, 800 mg. yeast. A, 600 mg. of a yeast differing in potency from that given to the other groups.

It is seen that the control animals in each group grew better than the rats receiving cod liver oil, and that the animals consuming the larger amount of cod liver oil grew the least. It becomes at once apparent, however, that no protective action can be assigned to yeast when given in the larger quantity, for although it is obvious that the growth responses in the groups subsisting on the cod liver oil diets were definitely

better when the yeast supplement was liberal, the growth of the control animals was improved to an equal degree.

Another group of animals (twenty-five in all, of which eleven were controls) received as supplement a Fleischmann yeast which was calculated to furnish not much more vitamin B but roughly twice the amount of vitamin G yielded by 400 mg. of the other yeast. This preliminary experiment was undertaken because of the suggestion arising from the work of De Vries and Puister ('33) and Bell, Gregory and Drummond ('33) that the harmful effect of 15% cod liver oil diets might be

TABLE 2

A comparison of the mean gains in weight and food intake of groups of rats fed diets containing cod liver oil and peanut oil

NUMBER OF RATS	DIETARY FAT	YEAST SUPPLEMENT	MEAN GAIN IN WEIGHT	PROBABLE ERROR	SIGNIFICANCE RATIO	TOTAL FOOD CALORIES [1]		
						Mean	Probable error	Significance ratio
		mg.	*gm.*					
8	27% CLO	400	217	10.0	3.8	3310	64	3.4
8	27% PO	400	258	4.4		3620	65	
8	27% CLO	800	293	9.0	3.2	4000	95	4.7
8	27% PO	800	344	13.5		4640	97	
14	27% CLO	600 [2]	257	3.8	6.9	3000	32	7.0
11	27% PO	600 [2]	301	5.2		3380	44	

[1] Includes those derived from the yeast.

[2] This was a different yeast. The experimental period for this group was 20 days shorter than for the preceding groups.

prevented by a liberal intake of vitamin G. The composite curves for these groups are also presented in figure 1 (A) and show that the difference in growth between the groups receiving cod liver oil and peanut oil is still apparent.

In order to ascertain whether these differences in body weight between the controls and their experimental mates were significant, and whether they could be correlated with the food consumption, the data were submitted to statistical analysis. A summary of the results from animals whose dietary fat was either entirely cod liver oil or peanut oil is given in table 2.

On comparing the significance ratios between the mean gains in weight and mean calorie intake, it is seen at once that the control animals grew better because they consumed significantly more food. With respect to its effect on growth of the albino rat, therefore, no direct toxic action was found attributable to the cod liver oil when given at 18% and 27% levels in the diet.

Observations on the growth of mice were incidental to the major study, i.e., the examination of the histologic preparations of mice killed at various periods. The interpretation of the composite curves presented in figure 2 is therefore complicated not only by discrepancies in the number of animals used in each group but also by the progressively diminishing numbers as the experimental period became greater. Nevertheless, it is apparent that 1) the animals subsisting on the diets containing cod liver oil did not grow as well as those fed rations in which peanut oil was used as the dietary fat, and 2) the growth was, in general, not improved by the addition of yeast in double the amount.

The question at once arose as to whether the differences in weight could be explained on the basis of differences in food consumption. It was very difficult to obtain accurate data on food consumption throughout the entire experimental period, as the mice spilled and scattered their food frequently. An average daily food intake was therefore calculated from a consideration of those periods in which the food could be measured with some degree of accuracy, assuming that the mice ate the same amount of food during the days when the quantity eaten could not be determined. On this basis it was calculated that the gains in weight made by the controls were approximately from 9 to 18% greater than could be accounted for by the differences in calorie intake.

It appears that cod liver oil at a level of 20% in the diet exerts an unfavorable effect upon the growth of the albino mouse. This confirms the statements of Agduhr ('27) that mice are more susceptible to the 'toxic' effects of cod liver oil than rats. However, it may be pertinent to point out that on

the basis of dosage per kilo body weight, the mice received significantly more cod liver oil than did the rats. Thus, as calculated from the daily food intake the dose for mice ranged between 20 to 22 cc. per kilo body weight per day. A similar calculation indicated that the average daily dose for the rat

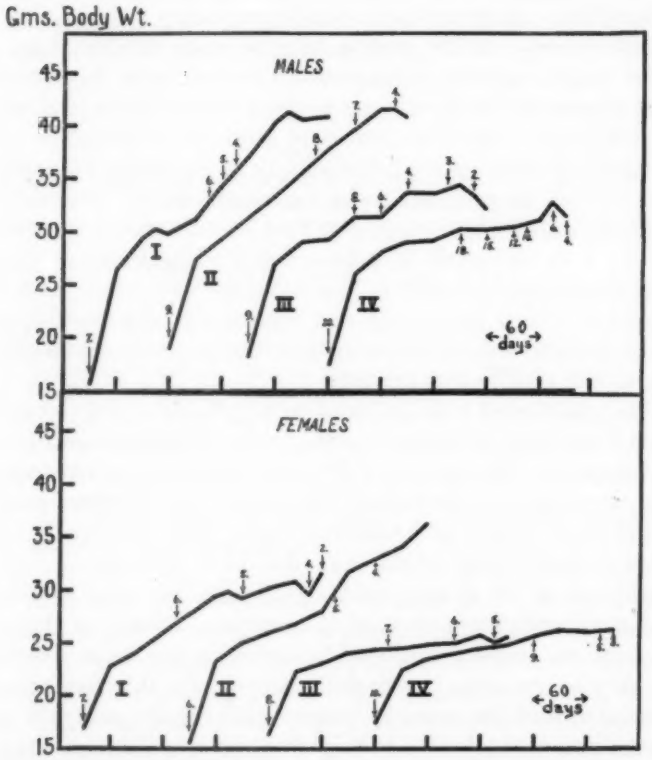

Fig. 2 Composite growth curves of mice representing the males and females of one of the strains used, showing a retardation of growth in the animals which consumed the diets containing cod liver oil. The number of animals progressively diminished because members of each group were killed at various intervals; the figures along the curves indicate the number of animals which furnished the data for the averages during the particular periods of the experiment. The levels of fat and yeast in the diets differed among the groups. These are designated as follows: I, 20% peanut oil and 20% yeast. II, 20% peanut oil and 10% yeast. III, 20% cod liver oil and 20% yeast. IV, 20% cod liver oil and 10% yeast.

was about 11 to 12 cc. per kilo body weight. It would not be profitable, therefore, to draw any conclusions from these data with regard to species differences.

Pathologic findings. Group I. Mice subsisting on a diet containing 20% cod liver oil and 10% yeast. This group included forty-two animals, thirty-four of which lived at least 150 days, and eight of which lived 300 days from the beginning of the experiment. The mice were killed at intervals and routine examination was made of the heart, liver, kidney and skeletal muscle.

Heart. Of the forty-two animals, five showed some degree of fibrosis, four of necrosis, and thirteen in all had variable amounts of pigment-containing mononuclear phagocytes in the myocardium (fig. 3, A and B).

Liver. Ten animals showed deposition of pigment in the liver in variable amount (fig. 3, C and D). In addition, four mice had focal accumulations of small mononuclear phagocytes in the liver.

Kidney. There were no findings of major interest in the kidney. Two animals showed lymphocytic accumulations in the renal cortex. The "calcareous incrustation in the region between cortex and medulla" as described by Andersson ('33) was not seen.

Skeletal muscle. In contrast to the extensive degeneration of voluntary muscle in herbivorous animals when these are given synthetic diets containing cod liver oil (Madsen, McCay and Maynard, '35), only six animals showed any changes in the muscle of the right hind leg. These changes consisted of an increase in sarcolemmal cells; in addition, muscle atrophy was seen in two animals (living 250 and 294 days, respectively, on the experimental régime). It is of interest that one mouse, which had yellow pigment deposits in both ventricles of the heart, had also an infiltration of pigment-bearing mononuclear phagocytes among the muscle fibers of the leg.

Spleen. Twelve animals had large spleens at necropsy. Microscopic sections of some of these showed the splenic cells to be loaded with finely divided fat. Numerous nests of large

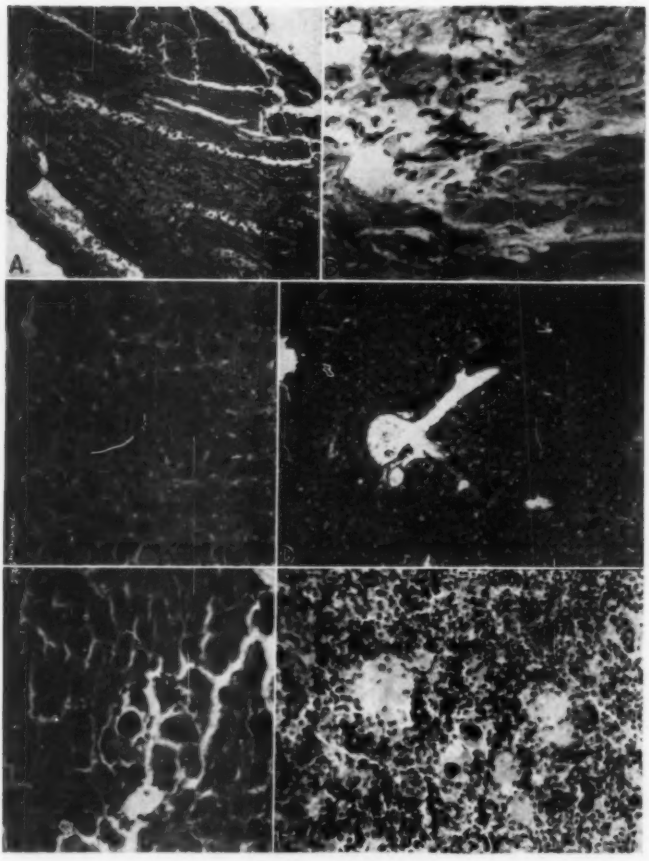

Fig. 3 A, mouse no. 72, group I. Acute and organizing myocarditis of right ventricle. H and E; × 125. B, mouse no. 84, group I. Monocytes containing brown pigment in scarred right ventricular myocardium. H and E; × 200. C, mouse no. 117, group I. Round cluster of liver cells containing brown, lipoid pigment. H and E; × 200. D, mouse no. 77, group I. Calcium salt deposits at sites of lipoid pigment in liver cells. H and E; × 125. E, mouse no. 67, group III. Multinucleated giant cells filled with lipoid pigment in liver. H and E; × 250. F, mouse no. 56, group I. Phagocytosed lipoidal material in mononuclear and multinucleated cells in spleen. H and E; × 200.

mononuclear cells were seen with phagocytosed lipoidal material. Also present were many multinucleated giant cells (fig. 3, F).

Group II. Mice subsisting on a diet containing 20% peanut oil and 10% yeast. Seventeen mice served as controls and were killed in groups with their experimental mates (group I). Of these seven animals lived 300 days after the beginning of the experiment.

Heart. No lesions were found in any of the animals.

Liver. Two of the animals which lived 300 days showed occasional yellow pigment deposits in the liver. Incidental findings were three cases of focal hepatitis, one of pericholangitis and one of a poorly circumscribed abscess.

Kidney. Focal lymphocytic infiltrations in the renal cortex were present in three animals.

Skeletal muscle. All the preparations showed normal architecture.

Group III. Mice subsisting on a diet containing 20% cod liver oil and 20% yeast. This group was composed of nineteen animals. All but one lived at least 150 days, and six were sacrificed after 300 days on the experimental régime. The procedure was the same as that described for group I.

Heart. Three of the nineteen animals showed myocarditis or myocardial fibrosis of mild degree. Four mice had pigment-bearing phagocytes in the myocardium.

Liver. Four animals showed deposition of pigment in the liver in variable amount (fig. 3, E). The livers of two animals contained occasional nests of small mononuclear phagocytes.

Kidney. Four animals had focal mononuclear cell infiltrations in the renal cortex. An incidental finding was a case of marked hyalinization of the glomeruli in an animal which had generalized subcutaneous edema at necropsy.

Muscle. In four mice there was evidence of proliferation of sarcolemmal nuclei, with some atrophy of the muscle fibers.

Spleen. Eleven animals had large spleens at necropsy. Examination of slides from the spleen of mouse no. 41 (see notes under heart and liver) gave a picture of enormous mononuclear cells loaded with lipoidal material. Present also was

a zone of necrosis in which calcium salts were deposited in the fatty material. There was actual bone formation in one zone.

Group IV. Mice subsisting on a diet containing 20% peanut oil and 20% yeast. Seventeen animals served as controls and were sacrificed in groups with their experimental mates (group III). Of these, six animals lived 300 days after the beginning of the experiment.

Heart. No lesions were found in any of the animals.

Liver. The liver of one animal contained a small spheroid zone of calcification (fig. 3, D). Other findings of incidental interest were two cases of focal hepatitis and one of hemangioma.

Kidney. In three animals there were focal lymphocytic infiltrations in the renal cortex. In one mouse a marked hyalinization of the glomeruli was noted.

Muscle. No changes were observed in the skeletal muscles of the right hind leg.

Group V. Rats subsisting on diets containing 27% fat. Twenty-seven rats were allowed to survive after the growth studies were completed. It was hoped to sacrifice them at the end of a year, but some of the animals had secondary infections, and these along with others in the group were necessarily killed earlier. Although the same tissues were studied as those of the mice, the only findings of interest were in the heart.

Five of the eighteen rats fed the diet containing cod liver oil showed necrosis and scar tissue formation in the myocardium, and in two of these, large mononuclear phagocytes were seen containing lipoidal material. It is to be noted, however, that three of these animals had acute infections elsewhere in the body.

Of nine animals serving as controls and subsisting on the diet containing peanut oil, three showed some fibrosis of the myocardium. Of these, one had an acute infection in the lungs.

It is difficult to interpret the results obtained in the rats, as three of the five animals in the cod liver oil series having heart lesions also had concurrent infection elsewhere. Furthermore, three of the controls had fibrotic changes in the myocardium, and of these one had an acute pulmonary infection. It is pertinent to point out that not one of the four animals which subsisted on the 27% cod liver oil diet for 350 days showed any pathologic changes.

COMMENT

Of fifty-two mice which subsisted on 20% cod liver oil diets for at least 150 days, fourteen animals showed in the heart necrosis, fibrosis and accumulations of monocytes with phagocytosed lipoidal material or pigment; of these only four showed as extensive lesions as are described by Agduhr (group I, nos. 72, 84, 33, 61). In the liver, fourteen animals showed changes, of which only six were significant; these were referable to degeneration of hepatic cells, the presence of pigment, multinucleated giant cells and mononuclear phagocytes, and to calcium salt deposition. Twenty-three of the mice had large spleens at necropsy. Unfortunately only a few microscopic preparations were studied, and these indicated fatty deposition of the parenchymal cells, active phagocytosis of lipoidal material by large mononuclear cells, the presence of multinucleated giant cells, necrosis and calcium salt deposition.

That the toxicity of cod liver oil must be considered apart from hypervitaminosis D has been discussed by Cox and Roos ('34). The fact that no calcium deposition was seen in the kidneys of the animals studied also makes it improbable that the changes were related to the intake of vitamin D; it has been pointed out by Steck and co-workers ('37) that the kidney is the most vulnerable of any tissue to the calcifying action of this vitamin.

It is not surprising that the feeding of cod liver oil to mice in a dose approximately 20 to 22 cc. per kilogram body weight per day should produce the effects described, in view of what

is known about the irritating action of cod liver oil. Pinkerton ('28), in studying the effect of intratracheal injection of oils, found that whereas olive oil did not appear to injure the lung in any way, cod liver oil produced a severe inflammatory reaction in the pulmonary parenchyma. Microscopically the alveoli contained enormous, bizarre-shaped giant cells and many large mononuclear phagocytes. Koehne and Mendel ('29) noted that when they administered cod liver oil parenterally the reaction at the site of injection was one of tissue necrosis, giant cell formation and elaboration of young capillaries. This was not seen with any other oil which they used. They make the comment:

> That the oil when so given has toxic properties is evident from the marked deterioration of the physical condition of all animals used and from the marked increases in nitrogen losses resulting in the dog. The body cells treat the injected oil or its emulsion as they would any irritating foreign substance, through the protective reticulo-endothelial system.

More recently Davson ('36) has shown that the favorable results which have been described to follow application of cod liver oil to wounds are not due to the presence of vitamin A (as has been suggested) but to the action of the oil in stimulating the production of granulation tissue.

The same type of proliferative reaction is apparently seen in some animals when large amounts of cod liver oil are ingested over a long period of time. It must be pointed out that in the present study cod liver oil furnished 40% of the calories consumed by the mice (32% and 47% of the calorie intake of the rats). Inasmuch as cod liver oil has been shown to be practically completely absorbed (Agduhr and Stenström, '30; Bell, Gregory and Drummond, '33), the constituents of the oil must have been present in the blood in high concentration during a relatively long experimental period. Under such conditions one might reasonably expect the pathologic changes to have occurred in a much greater percentage of animals than was actually found. In view of this, it is doubtful whether the feeding of amounts of cod liver oil near the therapeutic range would produce any observable injury.

It is of interest that no fatty livers were observed in any of the animals which were consuming large quantities of cod liver oil. The explanation undoubtedly lies in the fact that any effect which the cholesterol in the cod liver oil might have had in producing fatty livers was offset by the choline present in the yeast (Fletcher, Best and Solandt, '35) and the lipotropic factor contained in the casein (Best, Grant and Ridout, '36).

SUMMARY

Rats ingesting diets which contained 18% and 27% by weight of cod liver oil grew at a slower rate than animals receiving peanut oil as the dietary fat. These differences in growth, which were apparent even when the intake of yeast was liberal, were related to differences in caloric intake.

Mice given 20% cod liver oil as the sole source of fat did not grow as well as control animals receiving peanut oil. The presence of 20% yeast in the diet failed to exert any beneficial effect. In this species there was no correlation between body weights and food consumption.

Histologic examination was made of the heart, liver, kidney and skeletal muscle of animals fed diets containing either cod liver oil or peanut oil and sacrificed at various periods on the experimental régime. Some of the animals in the cod liver oil series showed pathologic changes in the heart, liver and spleen consisting of necrosis, fibrosis and phagocytosis of lipoidal material or pigment by monocytes and giant cells. These changes were not seen in any of the thirty-four mice serving as controls for this species, although among the rats three of the controls showed some fibrosis in the heart. It is pointed out that in view of the small percentage of animals which yielded changes in the organs as a result of cod liver oil feeding at high levels, the claims that cod liver oil in therapeutic doses can exert injurious effects are not substantiated.

Grateful acknowledgment is made to Prof. George R. Cowgill for his help and continued interest. It is a pleasure to acknowledge our indebtedness to Dr. Grover F. Powers of the

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METABOLISM STUDIES WITH RATS SUFFERING FROM FAT DEFICIENCY [1]

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SIX FIGURES

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INTRODUCTION

Rats suffering from fat deficiency (Burr and Burr, '29) show the peculiarity of consuming as much food as normal rats, and yet remaining small and finally becoming emaciated (Burr and Burr, '30). Furthermore, the deficiency symptoms are cleared up by the addition of small amounts of certain fats or fatty acids, and the rats resume growth. This raises a question as to the ability of these rats to synthesize fat, especially the essential fatty acids.

Wesson and Burr ('31) showed that following a carbohydrate meal the respiratory quotients of fat-deficient rats were well above unity, indicating ready synthesis of fat. The basal and assimilatory rates in the case of young rats in the early stages of fat deficiency were well above normal. This readily explained why the rats required the usual quantity of food although they were much smaller than the normal controls. Wesson and Murrell ('31) have extended this work and have shown a curative effect of the liquid fatty acid fraction of lard.

[1] Supported by grants from the medical research fund and general research fund of the University of Minnesota and from the National Live Stock and Meat Board.

For the above metabolism studies the rats were starved 14 hours and then fed sufficient dextrin for 12 hours of normal metabolism. This meant the ingestion of a large quantity of readily absorbed food in a few minutes, and although it gave an excellent comparison of the different rats, there was some doubt as to whether this procedure gave an insight into the normal behavior of the animals. The following experiments were designed to show how the rats differed from the normals when they were in their usual cage and on their regular diet.

APPARATUS

Since it was desirable to continue the studies for 24 to 48 hours, an open circuit apparatus was constructed. A constant speed, constant temperature spirometer drew a stream of dried outside air through the rat chamber. As the air left the chamber it was dried over sulfuric acid and calcium chloride. It then passed by a sampler which continuously aliquoted the air stream, and held the dry gas over dry mercury until time for analysis. The sampler is shown in figure 1. All the collecting vessels are full of mercury at the beginning of the run. The flasks are lowered by the constant speed windlass at any desired speed so that a sample may be collected in a few minutes or in several hours. The first collector begins after a preliminary period of about 30 minutes. Just as the first finishes the second begins to collect. All of the mercury receiving flasks are being lowered all the time, but the capillary tubes are so small as compared with the larger sample holder that 99% of the sample is collected as the flask passes the sample holder and only 1% collected in the capillary. After all samples are collected a correction may be applied, but it is too small to be considered in this work. The air passes over a capillary mercury trap which prevents any back diffusion of gases.

The collecting vessels hold about 125 cc. and are 4 inches long. The speed of the windlass was regulated for this work so that a sample was collected in 4 hours. Therefore, six collectors covered a period of 24 hours. Since these samples

are continuously aliquoted they represent the mean gas exchange for each 4-hour period and do not have the uncertainty in composition which is characteristic of an instantaneously collected sample.

The samples and spirometer are driven by the same shaft. The temperature of both remains the same and is constant to 1°C. Both get the same effect from barometric changes.

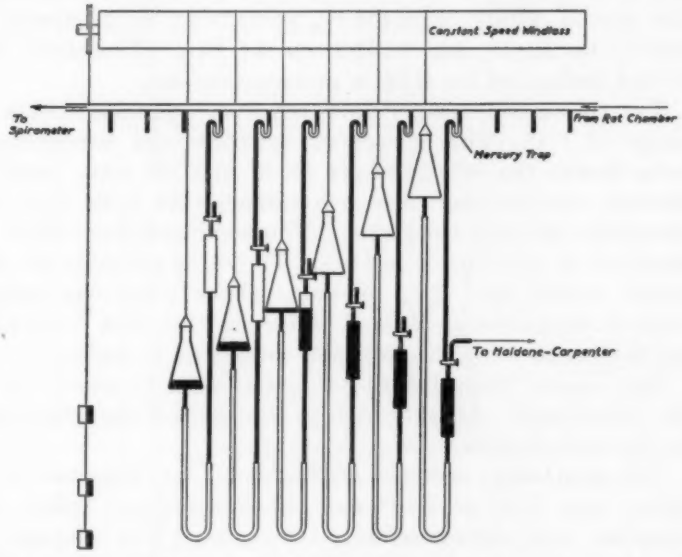

Fig. 1 A sampler which continuously aliquots the gas stream passing from the rat chamber to the spirometer.

Therefore, the same percentage of the air stream is being aliquoted at all times.

A chain of lead weights rises and counterbalances the mercury as it flows into the flasks. This keeps the windlass from becoming too heavily weighted on one side.

When it is time to begin the analyses the stopcocks are closed and the mercury flasks are hung on hooks so that the gas is under a slight positive pressure. A capillary tube leads

directly into an extra three-way capillary stopcock which is fastened to the Haldane-Carpenter. The tubes are washed out with the sample and the analyses for CO_2 and O_2 carried out according to Carpenter's (Carpenter, Fox and Sereque, '29) directions, all precautions being observed.

The spirometer has been set at such a speed that the maximum CO_2 is 1.6 volume per cent and most of the O_2 and CO_2 values remain above 1%. The Haldane-Carpenter has proved highly satisfactory, permitting an accuracy of 0.005% on the O_2 and 0.002% on the CO_2. Therefore, the second decimal of the R.Q. is probably correct.

The spirometer temperature remained constant within a range of 1°C. The barometric pressure and temperature being known the volume of air (at 0° and 760 mm.) passing through the chamber was determined, and from this the metabolic rate was calculated. The maximum fluctuation in speed of the spirometer is 1.5%. The rates probably are accurate within 2%. For quotients above unity the calorie value of oxygen at an R.Q. of 1.00 is used, with no correction for the excess CO_2. No nitrogen correction is made.

The colony temperature is maintained at 25.5°C. $\pm$ 1° the year round. All rats used in this work were acclimated to this temperature.

The metabolism chamber of thin metal was immersed in a water bath kept at 28.5° and the temperature inside the chamber was approximately the same. The change in temperature encountered by the rat on entering the metabolism chamber from the colony room was only 3°C.

PROCEDURE

All experimental rats were fed their daily supplements (vitamins, oils, etc.) about 4 o'clock each afternoon. Therefore this was considered a good time to start runs showing normal behavior. Three different studies were made. The results are summarized in figures 2, 3 and 4 and table 1 (A, B and C).

TABLE 1

Calories per individual rat are given for each 4-hour period. These calculations are independent of size.

PERIOD	1	2	3	4	5	6	7	8	9	10	11	12
A. Rats in figure 2												
Fat cures (average)	3.70	3.91	5.07	5.57	5.00	4.71						
Stock rats (average)	3.75	4.00	3.98	4.31	3.57	3.55						
Fat-starved (average)	4.01	4.1	5.51	5.68	4.78	4.77						
B. Rats in figure 3												
Fat cures (average)	5.31	5.70	5.17	4.67	3.87	3.63	3.95	4.51	4.50	4.21	3.80	3.61
Stock rats (average)	4.30	4.81	4.78	4.31	4.08	4.28	3.65	3.97	3.90	3.90	3.61	3.30
Fat-starved (average)	6.07	6.20	5.60	5.13	4.40	4.15	4.15	4.30	4.57	4.43	4.21	3.73
C. Rats in figure 4												
Stock rats (average)	4.31	4.68	4.88	4.57	3.93	3.60	4.55	5.40	5.33	4.51	4.03	4.07
Fat-starved (average)	6.13	6.27	5.75	5.10	4.35	4.78	5.60	6.10	5.35	5.28	4.61	4.18

One group (fig. 2) was starved 16 hours in the colony, transferred to the metabolism cage, and two samples were collected during the next 8 hours of fasting. The chamber was opened at 4 P.M. and the rats fed their yeast doses and other supplements, weighed cups of their usual diet put in

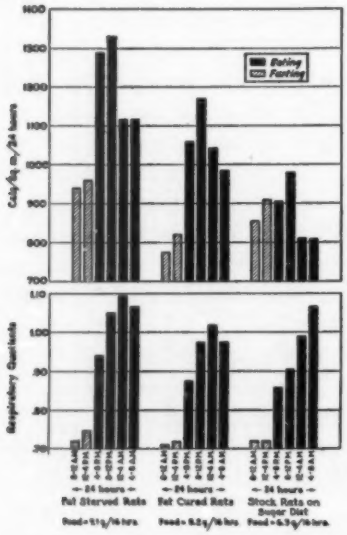

Fig. 2 Metabolic rates and respiratory quotients of three types of rats. Each column gives the mean value for a 4-hour period. Total time of each run is 24 hours. Rats were starved in colony cages 16 hours, transferred to the metabolism chamber and fasting values recorded for two periods (8 hours). The yeast dose and food were then put in the chamber and the values while eating were recorded for 16 hours.

and the run continued. A water bottle was always in the cage. The stock rats in this group were fed, during the run, the pure diet [2] mixed with 7% Northwestern dried yeast.

The next group (fig. 3) was not starved at all before the run. They were fed their supplements at the usual time (about 4 P.M.), immediately put into the chamber with their

[2] The basal fat-free diet fed in all this work consisted of: pure casein 12.0%; sucrose 84.1% and salts mixture 3.9%. This was supplemented by 0.7 gm. dried yeast and concentrates of vitamins A, D and E (Burr and Burr, '29).

usual diet and the gas exchange followed for 24 hours (six samples). Then the food cup was removed and the metabolism followed during 24 hours of starvation. Again, the stock rats were fed the pure diet plus 7% yeast instead of their usual stock diet.

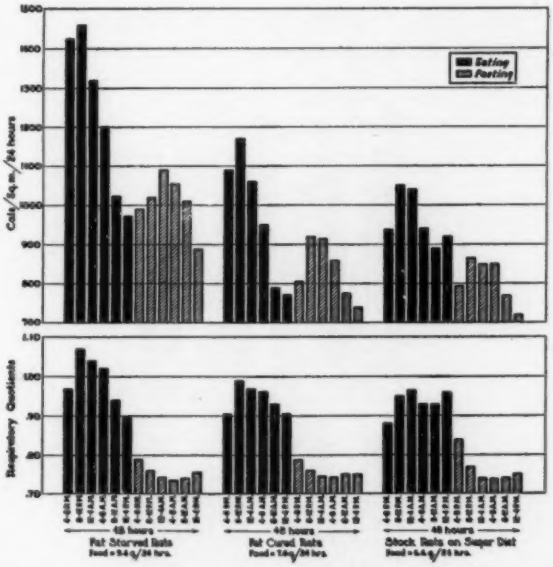

Fig. 3 Metabolic rates and respiratory quotients of three types of rats. Each column gives the mean value for 4 hours. Total time of run 48 hours. Food was in the cage during the first 24 hours (black column). Food was removed during the next 24 hours (shaded columns).

The third group (fig. 4) was studied for 48 hours without any starvation. Sufficient food for 48 hours was put in the cage. After the first 24 hours the chamber was opened, the supplements were fed and the run continued. In this case the stock rats were fed their usual stock diet. However, the chamber was opened and the food consumption measured at the end of 24 hours so that they were disturbed in exactly the same way that the fat-starved rats were.

Wesson and Burr ('31) noted that there was no greater activity by the fat-starved than by the other rats. The increased metabolic rate was shown by sleeping rats. However, some records of activity were made in connection with the present work. A typical 48-hour record is shown in figure 5. The activity at night is followed by long periods of rest during the day. On the whole, the records do not show that one type of rat was more active than another.

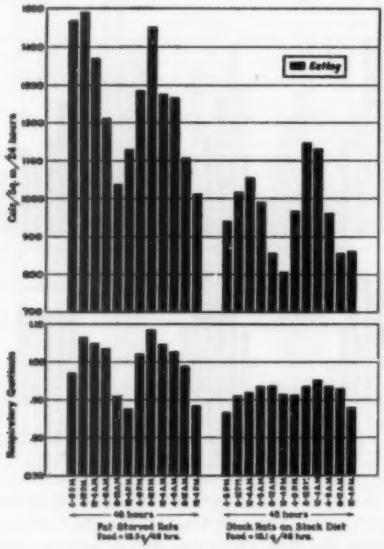

Fig. 4 Metabolic rates and respiratory quotients of fat-deficient and stock rats. The runs were continued 48 hours with food in the cage the entire time. The fat-deficient rats received the purified diet and the stock rats the usual stock diet.

Each line across the graph represents 2 hours, 15 minutes in time.

The records are made by suspending the cage on a spring so that each movement has a definite value in grams. The cage is built of extremely light material with a total weight less than that of the rat. This decreases the inertia and gives to the recorder a high sensitivity. The marks about 1 mm.

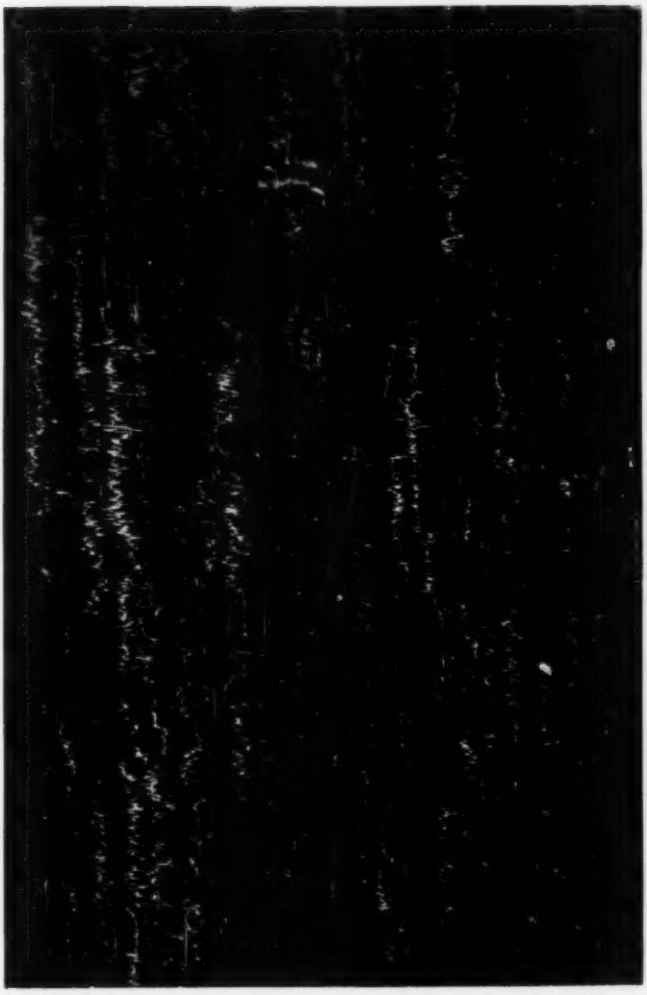

Fig. 5 Forty-eight-hour activity record of a fat-deficient rat. Food was in the cage throughout the run.

long at intervals in periods of rest are made by a slight movement of the head.

In this work the single formula of Lee and Clark ('29) was uesd for calculation of surface area ($S = 12.54 \times W^{0.60}$). As pointed out by Brody ('34) methods of measurement of surface are not satisfactory and heat production per unit of surface has no absolute meaning. However, the values given in figures 2, 3 and 4 are useful for comparison with data in the literature. The absolute calorie production by each rat over a period of 4 hours is given in table 1 (A, B and C).

RESULTS

The columns in figures 2, 3 and 4 give the average values of from four to eleven runs on as many different rats. The average food consumption per rat for the period during which food was in the chamber is given below each group. It was shown earlier (Burr and Burr, '30) that rats cured with fat consume the same amount of food as the fat-starved rats. But when these rats are put in the metabolism chamber at the higher temperature there is a tendency for the fat-starved rats to continue their usual consumption of food while the cures eat much less than normally. This is reflected in the high metabolic rates and high R.Q.'s. The high quotients and high metabolic rates reported by Wesson and Burr ('31) are confirmed. The addition of 10 drops daily of an oil has made the rats (fat cures) behave much more like the stock rats both with respect to quotient and rate of gas exchange. It is very interesting to see that when food is available the fat-starved rats maintain an average quotient above unity for as much as 16 hours out of 24 and in the second day of figure 4 this continued for 20 hours. If the high quotients are given the usual interpretation, i.e., fat synthesis, then it must be concluded that fat-starved rats readily synthesize fat. As has been pointed out in our earlier papers, analytical evidence as well as the curative effect of methyl linolate or linolenate indicate a very limited ability to synthesize these essential fatty acids. It would seem, therefore, that fat

synthesis from carbohydrate is largely confined to the more saturated acids.

The daily cycle of activity of all the groups is clearly shown in figures 3 and 4. The first column represents the period from 4 to 8 P.M. The metabolic rate reaches a peak from 8 P.M. to 4 A.M. and then falls to a low level during the day. This happens even during starvation as shown by the second day of figure 3. It is interesting that this is not associated with light since the chamber is almost totally dark.

There are some other facts of general interest in the field of metabolism. Periods of starvation of from 12 to 17 hours have been recommended in the study of basal metabolism (Benedict and MacLeod, '29). The starvation records in figure 3 show that a peak of metabolic rate is reached in 12 to 16 hours in all groups. Possibly this is due solely to increased activity because of hunger or because it is the middle of the night. The same curves should be run again with the starvation begun at another time of day. However, this metabolic curve as it stands indicates that the best chance of obtaining a short period basal on rats comes just after noon after a 24-hour starvation.

The R.Q.'s very uniformly reach a minimum at from 12 to 16 hours starvation during the period of maximum activity (fig. 3). It is interesting that the rats starved 18 hours in the colony and then put in the chamber (fig. 2) usually showed somewhat lower quotients than the rats starved in the metabolism chamber (fig. 3). There is an average difference of about 0.02.

The graphs show clearly that the utilization of carbohydrate as shown by high quotients does not greatly raise the metabolic rate. The stock rats of figure 2 show this most strikingly. They had been starved 18 hours in the colony room and transferred to the chamber. During the next 8 hours the average rates remained between 850 and 900 cals./sq.mm./24 hours. But as soon as they had eaten all they wanted and were asleep again the next day the rates fell to 800 calories (well below the starvation rates) while the quotients rose to values above unity.

On the other hand, the great rise of metabolic rate which occurs immediately after the feeding of the yeast dose at 4 P.M. indicates that the taking of this material into the stomach does greatly increase metabolism. It would seem, therefore, that the early digestive action on certain foods demands a great increase in metabolism while the absorption and utilization of carbohydrates resulting in high R.Q.'s produces no large increase in energy release. An estimate of the effect of food on metabolic rate during different periods of utilization can be made from the data in figure 3 if it is assumed that activity during each period of starvation is the same as for the corresponding period when food was in the

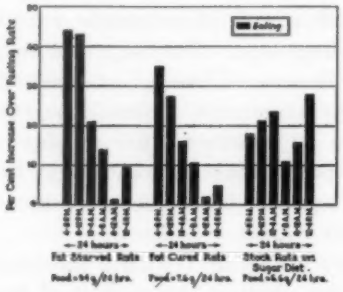

Fig. 6 The effect of food on metabolic rate expressed in percentage increase over the starvation rate. The values were obtained from figure 3 by subtracting column 7 from column 1, column 8 from column 2, etc.

cage. The percentage increase of rate with food in the cage over the rate during starvation is shown graphically for the six periods in figure 6. The fat-starved rats show an increase of 44% soon after receiving their yeast dose. The excess rate has fallen to 14% in the fourth period although the quotient is still well above 1.00. By 8 o'clock in the morning, the increase in rate has fallen to 1% while the quotient is 0.94. These high quotients indicate continued absorption of carbohydrate and probably fat synthesis. It appears, therefore, that the early digestive action on certain foods demands a great increase in metabolism while later utilization may be carried on at a basal level of energy exchange.

This is in accordance with two statements made by Lusk ('23): "It is, therefore, apparent that the intensity of metabolism is not related to the height of respiratory quotient;" and, "It happens frequently that with the cessation of glucose absorption the respiratory quotient remains at 1.00 indicating that carbohydrate is still the essential food, and yet the metabolism has fallen to the basal level."

Recent experiments have led to the view that Lusk's plethora theory as applied to carbohydrates must be abandoned or greatly modified (Wilhelmj, '35). The experiment here presented may be interpreted as giving evidence that the intermediates in the reaction carbohydrate→fat do not have a large S.D.A. Unlike the conversion of glucose to glycogen, the synthesis of fat goes spontaneously as an ordinary fermentation, and this reaction is not accompanied by any increased burning of carbohydrate as evidenced by intake of oxygen.

The very great increase in metabolic rate during the period of greatest food intake (from 4 to 12 P.M.) followed by an essentially basal level while the R.Q. is still high leads to the view that in carbohydrate foods the factors most important in raising the metabolic rate are to be found in water exchange (Carpenter and Fox, '30), work of digestion (Zuntz's Verdauungsarbeit) (Brody, '34) and glycogen synthesis (Wilhelmj, '35).

SUMMARY

Fat-deficient rats may synthesize much fat each day as indicated by high respiratory quotients.

The fat synthesized from carbohydrate does not contain appreciable quantities of the essential fatty acids since these must be added to the diet to prevent decline and death.

Although much smaller, the rats have a higher metabolic rate than their controls. Consequently, they have a much higher rate calculated as calories per square meter of surface.

A normal diurnal activity is shown for all groups, which is independent of light and food.

The minimum R.Q. is reached after 12 to 16 hours of starvation at 28.5°C.

Minimum metabolic rate is reached in the afternoon after 20 hours of starvaton.

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CONTROL FEEDING TECHNIC IN BONE CALCIFICATION STUDIES [1]

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Among the many factors known to affect the calcification of bone, the calcium content of the food has been shown to be of importance. Thus, by incorporating different quantities of this element into the ration Sherman and Booher ('31), Shohl and Wohlbach ('36), Brown et al. ('32), Bethke, Edgington and Kick ('33), Mendel ('34), and Fairbanks and Mitchell ('36) were able to alter the rate at which bone salts were stored by experimental animals. These findings point to the need for eliminating chances of a shifting intake of calcium, and possibly of phosphorus, in studies concerning the calcification of bone. A source of such an error is introduced under conditions of ad libitum feeding if the elements are incorporated into the ration at a constant percentage. This fact has been largely unappreciated, although, in studies in which two diets are being compared, it is not uncommon to find animals on the more satisfactory ration consuming twice as much food as those on the poorer ration. Even in vitamin D assay studies in which the amount of ash deposited in the bones has been suggested as the sole criterion, provision for the control of this factor has not been made (Sherman and Stiebeling, '29; Hart, Kline and Keenan, '31; Griem, '34).

[1] This paper appeared on the program of the annual meeting of the American Home Economics Association, June, 1933.

[2] A portion of these data was presented in partial fulfillment of the requirements for the degree of master of science at the University of Illinois by Lillian Merritt, June, 1932.

It is highly conceivable that much of the lack of uniformity in bone ash values observed by some investigators is due to differences in food consumption (and hence in mineral intake) as fostered by ad libitum feeding technic.

With this possibility in mind the present study was started in 1931. The data demonstrate that, on the ingestion of a ration adequate in all respects and containing a fairly generous percentage of mineral salts, the litter mate animal which consumes the more food will store the more ash in its bones. Two technics are described which have been successful in reducing such variations which might occur as a result of differences in appetite between animals being compared. One method involves the use of control feeding as sponsored by Mitchell ('29–'30), the other, the feeding of the calcium and phosphorus moiety of the mineral salts apart from the ration.

EXPERIMENTAL

The experimental animals were healthy, young albino rats taken from a colony that had been reared and maintained on an apparently satisfactory ration [3] for several years. The litters were reduced in number to eight young on the second day after birth and were taken from the mother on the twenty-first day. Female rats weighing at least 37 gm. at weaning were used. They were confined to individual cages with false bottoms to reduce coprophagy. The amount of light to which they were exposed was reduced to a minimum by placing them in a north basement room and by hanging a closely woven black cloth around the shelves on which the cages were kept.

For 1 week following weaning the rats were on a vitamin D-free ration to aid in the depletion of any possible stores of

[3] The ration for the breeding colony had the following composition: 2400 gm. ground whole wheat, 1200 gm. whole milk powder (Klim), 360 gm. ground meat scrap, 48 gm. C. P. NaCl, and 200 gm. brewers' yeast. Lettuce, in amounts of 40 gm., was given twice a week and the same amount of carrots once a week until parturition at which time they were withdrawn. During the periods of growth and rest between litters one-half of the whole wheat was exposed to ultraviolet light for a period of 30 minutes at a distance of 18 inches. During pregnancy and lactation the ration was not irradiated.

vitamin D. This ration had the following composition: low-ash casein (Swanson, '30) 18, starch 78, and salt mixture no. IV (Osborne and Mendel, '18) 4%. Yeast [4] and spinach powder [5] were fed daily at levels of 400 mg. and 50 mg., respectively.

For the experimental study three litter mate rats, as identical as possible in weight, appetite, and vitality, were selected to comprise a group. All animals of this triad were given the same basal ration plus the allotted supplements with the exception of vitamin D. Two of these animals were then chosen for a paired-feeding regimen in which the animal with the poorest appetite determined the quantity of basal ration consumed by both. One received cod liver oil [6] daily, the other, the same amount of corn oil [7] but no vitamin D. The third rat of the triad was fed the basal ration ad libitum and cod liver oil daily. The animals were kept on these diets for 28 days and hence, were 56 days old when the experiment was terminated.

The criterion adopted for the calcifying effects of the ration was the amount of ash deposited in the bones. The animals were sacrificed with ether and both femurs immediately excised. The technic used in the removal of the tissues and in the desiccation, extraction, and ashing of the bones has been fully described in another publication (Outhouse, Macy, and Brekke, '28). The ash content was calculated on the basis of the fat free, moisture free femur.

Series 1. The ration was essentially that advocated by Sherman and Stiebeling ('29) for the quantitative estimation of vitamin D and consisted of the following: low-ash casein 18, starch 78, and salt mixture no. IV (Osborne and Mendel, '18) 4%. Daily adjuvants for each animal of the triad were 400 mg. of yeast and 50 mg. of spinach. These were fed apart

[4] Dried brewers' yeast purchased from the Northwestern Yeast Company, Chicago.

[5] Powdered spinach prepared by the California Vegetable Concentrates, Inc., Los Angeles, California.

[6] Sample no. 156 of cod liver oil obtained from the E. L. Patch Company, Stoneham, Massachusetts.

[7] The commercial brand, Mazola, was used.

from the ration in order to insure sufficient vitamins A, B, and G for growth, irrespective of the amounts of basal food eaten. Cod liver oil, in a 50 mg. portion, was fed daily to one of the pair receiving identical amounts of the basal ration and to the other member of the triad eating ad libitum.

Series 2. In this series the calcium and phosphorus moiety of the salt mixture was fed separately from the basal ration which consisted of low-ash casein 18, starch 79.4, and salt mixture no. XXX [8] (Osborne and Mendel, '26–'27) 2.6%. The daily adjuvants for each member of the triad were 400 mg. of yeast, 50 mg. of spinach powder, and 287 mg.[9] salt mixture no. IV. As in series 1, two animals of the group were given 50 mg. of cod liver oil daily, whereas the third rat received no vitamin D. In order to insure the ingestion of the mineral salts the dry adjuvants were mixed together with a small portion of the basal ration. The remainder of the ration was withheld until complete consumption of the supplements occurred.

DATA AND DISCUSSION

Series 1. The data for food intake, weight gains, and bone ash for the fifteen triads in this series are presented in table 1. The amount of food eaten by the rats on paired feeding ranged from 134 to 229 gm. for the 28-day period. In each case the animal without added vitamin D had the poorer appetite and hence, limited the amount of food eaten by its pair mate. In every triad the rat on the ad libitum feeding plan consumed considerably more food than did its litter mate on paired feeding; the total increase ranged from 38 to 137 gm.

The weight gains made by the rats receiving no vitamin D were variable with a minimum of 29 and a maximum of 67 gm.

[8] The salt mixture no. XXX is free of calcium and phosphorus; at a level of 2.6% the mixture furnishes the elements other than calcium and phosphorus in approximately the same amounts and proportions as would be supplied by 4% of the Osborne and Mendel salt mixture no. IV.

[9] This quantity of salt mixture contained the same amount of calcium as was found in 30 cc. of whole milk. This level of milk was shown, in a preliminary study, to induce normal calcification in bones of rats fed the ration as used in this series.

for the 28-day period. Their pair mates receiving cod liver oil attained greater weights. The increase was slight; but it appeared in thirteen of the fifteen triads and was found, on analysis of the data (Student, '15–'17), to be statistically

TABLE 1

Response of litter mate groups to the ad libitum ingestion of a mineral-rich basal ration

LITTER MATE NUMBERS	PAIR WITH SAME INTAKE OF BASAL RATION					LITTER MATE FED BASAL RATION AD LIBITUM AND VITAMIN D		
		Rat fed no vitamin D		Rat fed vitamin D[1]				
	Food intake (28 days)	Gain in weight (28 days)	Ash content of bone[2]	Gain in weight (28 days)	Ash content of bone	Food intake (28 days)	Gain in weight (28 days)	Ash content of bone
	gm.	*gm.*	%	*gm.*	%	*gm.*	*gm.*	%
2602–04[3]	207	58	52.3	55	59.4	262	81	60.7
2605–08[3]	181	40	52.8	50	59.6	237	85	60.6
2634,35,39	168	42	51.7	60	55.2	305	80	59.2
2636–38	180	40	47.4	58	55.0	309	80	59.2
2696,97,2701,02[3]	229	53	56.3	60[4]	58.6[4]	295	86	60.1
2698,99,2700[3]	212	60	49.7	65	59.0	275	85	59.5
2802–06	203	54	55.9	67[4]	60.4[4]	254[4]	80[4]	61.1[4]
2807–09	222	67	56.9	67	60.2	260	84	61.4
2810–12	134	29	53.6	37	57.1	266	82	60.3
2813–15	174	46	46.4	53	56.8	248	76	59.7
2872,76,77[3]	173	36	52.2	49	58.4	270	95	60.8
2873,75,78[3]	181	41	50.5	52[4]	57.4[4]	243	83	58.9
2893–95	182	45	49.4	49	58.6	259	84	57.7
4449,50,54	143	39	50.1	41	57.2	224	70	60.9
4497–4500	144	39	50.3	41	56.4	229	75	59.1
Average	182	46	51.7	54	58.0	262	82	60.0
Standard deviation	27.6	10.0	3.0	9.1	1.6	24.7	5.5	1.0
P.E.	18.6	6.7	2.0	6.1	1.1	16.7	3.7	0.7

[1] Fifty milligrams cod liver oil daily.

[2] Calculated on basis of dried, alcohol-ether extracted bone.

[3] Litter mate groups.

[4] Average of two rats.

significant as shown by odds greater than 9999 to 1. The inability of this group to make greater gains was due, in large part, to the restricted food supply. This was evidenced by the fact that the litter mates fed cod liver oil and eating ad libitum grew decidedly better.

The ash content of the bones of the animals on any one of the dietary modifications showed considerable variation as seen in table 1. In comparing the rations, however, significant differences are in evidence, whether the data be considered for each triad or as an average for all the members on a given regimen. As might be expected, in each of the fifteen triads the animal receiving cod liver oil had an appreciably greater bone ash content than had the other member of the pair receiving the same amount of food. The difference between 58.0 and 51.7% ash for the rats receiving cod liver oil and no cod liver oil, respectively, represents an average increase of 12% in the relative amount of ash deposited in the bones. This increase is definitely attributable to the vitamin D in the cod liver oil. Of the two animals receiving this adjuvant, the one consuming the ration ad libitum had the more ash in its bones in fourteen of the fifteen triads. On statistical analysis of the differences, odds greater than 9999 to 1 were found, indicating practical certainty that the results were not fortuitous.

Inasmuch as the only difference between the diets of the two animals receiving cod liver oil was the quantity of basal ration consumed, one may conclude that the increase in bone ash was due to the greater quantity of food ingested by the animal on the ad libitum regimen. This series demonstrates, then, that with mineral rich rations a disparity in the amount of food consumed by animals being compared can bring about a difference in the amount of ash deposited in the bones. With this type of ration, therefore, a plan of control feeding should be instituted in studies in which bone ash values are used as the sole criterion.

Series 2. In this series the possibility of a difference in the rate of calcification being induced in animals as a result of unequal appetites was decreased by feeding the calcium and phosphorus portion of the mineral salts in a constant daily amount separate from the ration. The results for the sixteen litter mate triads are tabulated in table 2. The amount of food eaten by the animals on the paired feeding plan varied

from 112 to 206 gm. for the 28-day period. As in series 1, the rat receiving no cod liver oil had the poorer appetite of the pair and hence, limited the amount of food that its pair mate received. The litter mates on the ad libitum regimen

TABLE 2

Response of litter mate groups to the ad libitum ingestion of a basal ration deficient in calcium and phosphorus

LITTER MATE NUMBERS	PAIR WITH SAME INTAKE OF BASAL RATION					LITTER MATE FED BASAL RATION AD LIBITUM AND VITAMIN D		
		Rat fed no vitamin D		Rat fed vitamin D[1]				
	Food intake (28 days)	Gain in weight (28 days)	Ash content of bone[2]	Gain in weight (28 days)	Ash content of bone	Food intake (28 days)	Gain in weight (28 days)	Ash content of bone
	gm.	*gm.*	%	*gm.*	%	*gm.*	*gm.*	%
3246–48	206	53	54.5	67	58.7	223	82	56.9
3801–05	137	23	55.5	30[3]	59.2[3]	213[3]	69[3]	60.0[3]
3806–09	193	44	52.5	50	57.1	219[3]	70[3]	58.1[3]
3831,33,35[4]	165	33	54.9	36	59.0	242	83	60.1
3832,34,36[4]	167	47	53.9	47	58.5	232	81	59.9
3826–29	173	30	57.1	29	61.7	248[3]	80[3]	60.5[3]
3837–40	175	36	57.6	58	61.2	203[3]	88[3]	59.0[3]
3862–64	161	46	53.2	57	60.0	232	78	60.1
3867–69	161	44	53.2	45	58.2	232	82	58.5
4393–95	134	36	54.2	35	58.8	226	81	59.5
4396–98	141	31	51.4	34	58.8	243	80	58.1
4387,90,91[4]	112	20	52.4	20	57.6	215	75	59.3
4388,89,92[4]	112	15	52.4	12	59.1	193	54	60.2
4384–86	129	31	50.9	31	57.8	236	82	58.8
4408–10	117	31	52.8	35	56.4	191	82	57.8
4498–4502	112	21	50.9	20	55.1	244	77	55.4
Average	150	34	53.6	38	58.6	225	78	58.9
Standard deviation	28.8	10.5	1.9	14.6	1.6	17.2	7.7	1.3
P.E.	19.4	7.1	1.3	9.8	1.1	11.6	5.2	0.9

[1] Fifty milligrams cod liver oil daily.

[2] Calculated on basis of dried, alcohol-ether extracted bone.

[3] Average of two rats.

[4] Litter mate groups.

in all cases had greater avidity for food. In general, however, the food intakes were appreciably lower than in series 1, in which 73% of the animals consumed 248 gm. or more of food. None of the rats in series 2 partook of more than this

amount. The cause of this poor food intake was not determined. It was appreciated during the experiment, however, that the animals did not particularly relish the mixture of adjuvants containing the inorganic salts. Inasmuch as the basal ration was withheld largely until the supplements had been consumed, it is possible that the animals did not have sufficient time in which to obtain an adequate amount of the basal ration.

The gains in weight made by the animals without cod liver oil were not very satisfactory, averaging 33.8 gm. for the 28-day period. The addition of cod liver oil to the ration stimulated growth above that in the pair mate in nine of the sixteen cases. The chances that this increase was not accidental were found on statistical analysis to be 57 to 1. The member of the triads eating ad libitum and receiving cod liver oil, however, gained, as an average, more than twice as much as either of the paired animals. The magnitude of their gains was similar to that observed for series 1.

The relative amount of ash laid down in the bones of the animals receiving no cod liver oil showed a range from 50.9 to 57.6% and averaged 53.6. In every one of the sixteen cases the feeding of vitamin D to the pair mate resulted in a definitely greater amount of bone ash—the data ranging from 55.1 to 61.7%. The average value of 58.6% represents an increase in bone ash of 9.3% above that obtained for the group not receiving cod liver oil. For the two groups receiving cod liver oil, the average bone ash values for the ad libitum fed group were almost identical with those for the group on restricted feeding; i.e., 58.9 and 58.6%, respectively. In considering the data triad by triad, there were four cases in which the restricted rat had the higher bone ash, nine in which this rat had the lower amount, and three in which the values were practically identical. Although the preponderance of data might appear to indicate that the animals on the ad libitum regimen had the higher content of ash, statistical analysis showed that the odds were only 7 to 1 that the differences were significant.

It is obvious from this study that the additional amount of the basal ration as consumed by the ad libitum fed rats did not result in calcification beyond that obtained in their litter mates on restricted food. One may conclude, then, that when fairly generous amounts of calcium and phosphorus are fed daily apart from the ration a difference in the quantity of a calcium poor food eaten by the animals being compared is of minor consequence insofar as bone calcification is concerned. Under the conditions established in this regimen, therefore, a system of control feeding need not be adopted.

TABLE 3

Comparison of the average daily consumption of calcium and phosphorus in series 1 and 2

	SERIES 1		SERIES 2	
	Paired-feeding groups	Ad-libitum group	Paired-feeding groups	Ad-libitum group
	gm.	gm.	gm.	gm.
Basal ration, average daily intake	6.5	9.4	5.3	8.0
Calcium—in basal ration, average daily intake	0.033	0.048	0.001	0.001
Calcium—in adjuvants, daily intake	0.001	0.001	0.038	0.038
Calcium—total, average daily intake	0.034	0.049	0.039	0.039
Phosphorus—in basal ration, average daily intake	0.027	0.040	0.009	0.013
Phosphorus—in adjuvants, daily intake	0.007	0.007	0.028	0.028
Phosphorus—total, average daily intake	0.034	0.047	0.037	0.041
Calcium: phosphorus ratio	1: 1	1: 0.95	1: 0.96	1: 1.06

Comparison of the two technics. As seen in table 3, the average intake of calcium and phosphorus eaten by the animals in the two series was almost identical except for the ad libitum group in series 1. These latter animals consumed, as a daily average, 14 mg. calcium and 12 mg. phosphorus more than did their litter mates on paired feeding. This increased consumption of these bone calcifying elements must be considered responsible for the increase (3.4%) in bone ash of this group over that of their litter mates receiving cod liver oil and a restricted amount of food. For series 2, although the ad libitum group ate, as an average, 2.7 gm. more of the basal ration daily than did their litter mates on paired feeding, the

average intakes for both groups were practically identical in respect to calcium and nearly so for phosphorus (the inclusion of a low-ash casein in the ration made this possible). This similarity in the amount of these elements consumed by both groups is reflected in the lack of difference in bone ash values of the two rats of the triad receiving cod liver oil.

A consideration of the bone ash values for the rats of the two series indicates that the two rations were similar in respect to calcifying properties. For the animals receiving no vitamin D averages of 51.7 and 53.6% ash were obtained for series 1 and 2, respectively. For the pair mates receiving cod liver oil, the averages were practically identical for the two series; i.e., 58.0 and 58.6%. A striking resemblance also is seen between these values and that of 58.9% for the litter mates eating ad libitum in series 2.

The adaptability of the two technics to vitamin D assay work in which bone ash values are used as a criterion for calcification should be considered. For calcium-poor foods, which could be administered in a large quantity without affecting the ratio or total intake of calcium and phosphorus, the regimen as used in series 1 would be recommended because of ease of administration of both adjuvants and basal ration. If calcium rich foods were superimposed on this ration, the resulting change in level and ratio of salts might induce calcification which should not be attributed to vitamin D activity. With such foods ration 2 is advocated; since, by modifying the salt adjuvant, the desired ratio and level of calcium and phosphorus may be maintained, even though different quantities of the same food are being fed. This technic was used successfully in the determination of vitamin D potency of milk, a study which will be reported soon.

CONCLUSIONS

Data are presented which indicate that, in studies on bone calcification, it is desirable to equalize the mineral intake of the animals being compared. Two technics have been presented whereby such control is possible. One procedure

involves the paired-feeding method, the other provides for the feeding of the bone calcifying salts separate from the ration. The application of these technics to vitamin D assay studies in which bone ash values are used as a criterion is discussed.

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A COMPARATIVE STUDY OF THE GROWTH-PROMOTING AND BONE-CALCIFYING EFFECTS OF SEVERAL CARBOHYDRATES [1]

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ONE FIGURE

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The suggestion that lactose might be unique among other carbohydrates was mentioned by Mathews ('21) who noted its universality in the food provided by nature for all mammals during the period of greatest galactolipin synthesis. Since then numerous investigators have compared the physiological effect of diets containing milk sugar with those containing other naturally occurring carbohydrates. The problem has been approached from many angles. Irrespective of the criterion used, the action of lactose has been reported as exhibiting a lack of conformity with that of other carbohydrates, with the possible exception of galactose. One is led to conclude that these two sugars have a physiological role not participated in by other saccharides.

This paper deals with studies which were started in 1931. The data corroborate the previously reported stimulating action of lactose on the calcification of bones but do not support the contention that this carbohydrate has a specific effect on growth. A subsequent paper will deal with mineral metabolism as influenced by lactose and other carbohydrates.

[1] A portion of these data was presented before the annual meeting of the American Association of Dairy Science, June, 1933, Urbana, Illinois.

EXPERIMENTAL

The animals used in this experiment were healthy, 21-day-old albino rats which had been selected from the stock colony of the nutrition laboratories of the Home Economics Department. The ration of their progenitors, their age and weights at weaning, and the conditions under which they were caged during the experiment are discussed in detail in a previous publication by Outhouse, Smith and Merritt ('37). The assessment of the growth induced by the various rations was made on the basis not only of gains in body weight over the experimental period but also of body length. The latter value was determined at the end of the experiment by measuring the distance between the nose and anus of the etherized animals and represents final body length rather than gain in length. The criterion adopted for the study of the bone-calcifying action was the amount of ash deposited in the bones of the rats after 28 or 35 days on the rations. The data are expressed in terms of the percentage of ash in the fat-free, moisture-free bone. The methods used in preparing the bones for ashing are discussed in a publication by Outhouse, Macy and Brekke ('28).

The sugars fed were of C.P. grade.[2] The lactose was the type which naturally occurs in milk, and the galactose was the d-isomer. Neither gave a test for sterols; hence, they were not extracted with fat solvents. The lactose was free from ash; but the galactose, upon analysis, was found to contain 0.16% of calcium. The extra amount of this element consumed by the animals on the galactose supplement would approximate 1 mg. daily.

The quantity of lactose fed was determined on the basis of the following consideration. In earlier unpublished experiments 30 cc. of cow's milk daily was found to be the minimum quantity which, in the absence of cod liver oil, would insure normal calcification in rats. Therefore, an amount of lactose equivalent to that occurring in this quantity of milk

[2] Bakers-analysed lactose and sucrose were used and the galactose was a Pfanstiehl preparation.

was selected as the minimum for series 1, in which lactose was fed as a daily supplement. Animals receiving this level (i.e., 1.2 gm. daily) consumed, as an average, 23% of their calories in the form of lactose. For series 2 and 3, lactose was mixed with the ration at a level of 25%. In this case it contributed 26% of the total calories. The feeding of galactose was carried out at only one level; i.e., 0.6 gm. daily, a quantity which is equivalent to 95% of the galactose content of 1.2 gm. lactose.

Series 1. In this series, as in series 2, female rats were prepared for the experiment by depleting their bodies of whatever stores of vitamin D they may have had at the end of the nursing period. The ration used for this purpose consisted of a low-ash casein (Swanson, '30) 18, salt mixture no. IV (Osborne and Mendel, '18) 4, and starch 78%. Powdered spinach [3] and dried brewers' yeast [4] supplied all the necessary vitamins with the exception of vitamin D. After being fed this ration for 7 days, the animals were then selected for litter mate groups on the basis of weight and given the experimental ration for the following 28 days.

The experimental regimen employed consisted in feeding ad libitum a ration poor in calcium and phosphorus, together with daily supplements of a calcium- and phosphorus-rich mineral mixture and the necessary vitamin-carrying foods. The basal ration was composed of low-ash casein 18, starch 79.4, and salt mixture XXX 2.6%. The minerals necessary for the calcification of bones were supplied in the form of salt mixture no. IV at a level of either 287 mg. or 384 mg. daily. Vitamins A, B and G were supplied daily by 50 mg. of powdered spinach and 400 mg. of dried brewers' yeast. This ration was used in a previous study by Outhouse, Smith and Merritt ('37) and was found to support satisfactory calcification in the presence of vitamin D. Three litter mate rats were used in each group. All were fed the above ration and supplements.

[3] Powdered spinach was purchased from the California Vegetable Concentrates, Inc., Los Angeles, California.

[4] Dried brewers' yeast was obtained from the Northwestern Yeast Company, Chicago, Illinois.

One rat received no additions, one was given lactose or galactose, and the third rat was fed a 50 mg. dose of cod liver oil daily.[5] Lactose was fed at two different levels; i.e., 1.2 gm. and 2.0 gm. daily. In order to secure complete ingestion of the salt mixture and sugars, all adjuvants were mixed together with a small portion of the basal ration and placed in the cage early in the morning. When these were eaten, the basal ration was given ad libitum.

Series 2. In this study lactose was compared with starch, sucrose and cod liver oil. The carbohydrates and inorganic salts were mixed into the ration at a constant percentage. Four female rats, depleted of vitamin D and approximately identical in weight, were selected from the same litter. Four modifications of the same diet were prepared. The basal ration was complete in all necessary factors except vitamin D and had the following composition: low-ash casein 18, starch 67, salt mixture no. IV 4, yeast 10, and spinach 1%. When sucrose or lactose was fed, it comprised 25% of the diet and replaced an equivalent amount of starch. One rat was given the above ration; one the same ration plus 50 mg. cod liver oil daily; a third received the ration containing 25% lactose; and the fourth rat was fed the one containing 25% sucrose. These rations will be referred to as the starch ration, the cod liver oil ration, the lactose ration, and the sucrose ration, respectively. In order to avoid differences in intake of calories and mineral salts, a system of control feeding was adopted. All animals were allowed the same quantity of food; that one having the poorest appetite limited the amount that the others received. In order to equalize the caloric intake of all four animals of the group, the three which did not receive cod liver oil were given the same quantity of corn oil.[6] The experimental period lasted 28 days.

Series 3. In this study the technic differed from that in the preceding series in that male rats were used and the preliminary vitamin D-depletion period was not employed. The

[5] The E. L. Patch Company, Stoneham, Massachusetts, supplied the cod liver oil.
[6] Mazola oil was used.

animals, at weaning, were placed on the experimental rations and were maintained on them for 5 weeks. The age of the animals at the end of the period was 56 days the same as in series 1 and 2. Lactose and sucrose only were compared. The carbohydrates were mixed into rations which had the following composition: low-ash casein 18, starch 47, salt mixture no. IV 4, yeast 6, and lactose or sucrose 25%. Carotene,[7] administered daily in $\frac{1}{100}$ mg. doses, replaced spinach as the source of vitamin A. The paired-feeding technic was employed.

DATA AND DISCUSSION

The data accumulated in these three series are tabulated in tables 1 to 4 and will be discussed collectively. The rats apparently tolerated the doses of lactose and sucrose well as judged by the fact that there were no evidences of diarrhea or digestive disturbances at any time. However, the feces of the lactose-fed animals were bulkier than those excreted on the other rations. In practically all of the litter mate groups the animal eating the sucrose ration had the poorest appetite. In series 3 it was this rat which limited the quantity of food consumed by the pair. In series 2 the food alloted to the quartet was frequently determined by the sucrose-fed rat and just as often by the one on the starch ration.

INCREMENT IN SIZE

As influenced by lactose. The growth response of the rats to lactose was found to be dependent, to a considerable extent, on the type of feeding employed. Under conditions of ad libitum technic the rats fed this disaccharide made greater gains than did their litter mates on the basal ration in two of the three experiments (table 1). Under conditions of isocaloric feeding, however, the ingestion of lactose did not induce greater growth, as assessed by weight gains or by final body length, than was found in litter mates receiving

[7] Crystalline carotene, purchased from the S. M. A. Corp., Cleveland, was dissolved in Wesson oil.

TABLE 1

Average values for food intake, gain in weight, and bone ash of rats in series 1

EXPERIMENT NO.	CARBOHYDRATE		SALT MIXTURE NO. IV DAILY	NUMBER OF GROUPS	GROUPS FED BASAL RATION			GROUPS FED BASAL RATION AND COD LIVER OIL			GROUPS FED BASAL RATION AND CARBOHYDRATE		
	Kind	Amount daily			Food intake (28 days)	Gain in weight (28 days)	Bone ash[1]	Food intake (28 days)	Gain in weight (28 days)	Bone ash	Food intake (28 days)	Gain in weight (28 days)	Bone ash
		gm.	*mg.*		*gm.*	*gm.*	%	*gm.*	*gm.*	%	*gm.*	*gm.*	%
1	Lactose	1.2	287	8	153	33	50.1 ± 1.9[2]	234	69	57.9 ± 1.5	144	41	54.7 ± 2.2
2	Lactose	1.2	384	9	166	33	55.2 ± 2.2	218	62	60.9 ± 1.4	146	34	58.4 ± 2.0
3	Lactose	2.0	384	9	167	31	56.0 ± 2.1	211	55	61.7 ± 1.2	138	41	60.0 ± 1.0
4	Galactose	0.6	287	11	157	33	55.6 ± 1.9	226	71	60.3 ± 1.8	153	35	56.5 ± 1.6

[1] Calculated on basis of dried, alcohol-ether extracted bone.

[2] Standard deviation.

non-lactose rations. This was true for series 2 in which the lactose ration was compared with the starch, the sucrose, and the cod liver oil rations and in series 3 in which lactose was compared with sucrose. Statistically significant differences (Student, '15–'17) were not found on considering the data from the standpoint of averages for the groups or of differences between the pairs. The slight superiority in weight gains by the lactose-fed rats over those of litter mates on the basal ration in experiments 1 and 3 in series 1, as mentioned above, can therefore be explained on the basis of a greater calorie intake.

As influenced by sucrose. This disaccharide, when compared with starch or lactose, was found to have no effect whatsoever on weight gains or final body length of female rats in series 2 or on weight gains of the male rats in series 3.

As influenced by galactose. The growth promoting properties of galactose were observed only under conditions of ad libitum feeding and at a single level; i.e., 0.6 gm. daily. As seen in experiment 1 (table 1), this monosaccharide had no influence on either appetite or gain in weight in addition to that observed in litter mate animals receiving the basal food.

As influenced by cod liver oil. In both series in which cod liver oil was fed, the greatest gains were made by the animals receiving this supplement. In series 1, however, the strikingly accelerated growth was accompanied by a greater food intake and should be considered as being due primarily to this. Conclusive proof that some of this gain may have been the result of the ingestion of cod liver oil, is given in series 2. All members of the quartet received the same quantity of food, yet the animal on the cod liver oil ration gained more than the one on the starch ration in sixteen of the twenty groups. A statistical analysis of these data yielded chances of 9999 to 1 that the differences were not accidentical. Odds of a similar magnitude were obtained for differences in final body length of these animals.

These data show the growth promoting value of cod liver oil when added to these rations. In addition, they are of

primary significance in that they demonstrate that, if rations are not equal in their growth stimulating qualities, the method of control feeding can be relied upon to indicate that difference.

Comparison of data with those of other investigators. The results obtained in this study are not greatly variant from those reported in the literature. In most of the investigations in which ad libitum feeding technic was used the various authors have interpreted their data as indicating a difference between the growth promoting properties of lactose and other carbohydrates fed. The inclusion of milk sugar in a variety of rations was felt by several to be especially productive of growth (Kline, Keenan, Elvehjem and Hart, '32; Whittier, Cary and Ellis, '35). On the other hand, Jarvis ('30) and Koehler and Allen ('34) found lactose inferior to sucrose as a growth stimulator. Similar results were obtained by Whittier, Cary and Ellis ('35) when adult animals were used. Mitchell ('27) reported that rations containing more than 30% of lactose induced less growth than did those containing comparable amounts of starch, dextrin, maltose, or sucrose. In later work Mitchell and Dodge ('35) found that gains were subnormal on lactose containing diets only when this disaccharide comprised 70% of the ration. In none of these studies have food intakes been reported. With the exception of Whittier, Cary and Ellis ('35) no one has suggested that the differences in weight gains may have been due to a possible disparity in the amount of food consumed by the animals being compared. When these authors fed lactose under conditions of control feeding there was found only slight superiority of lactose over sucrose. If an incongruity exists between their results and those reported in this paper, it may be due to differences in the quantities of lactose fed. Ariyama and Takahasi ('29), feeding rats 30% carbohydrate in iso-caloric rations, observed greater growth with lactose than with sucrose and less than with starch. The slight differences observed and the small number of animals used should make any conclusions of dubious value. Certainly the data herein

reported for weight gains in the fifty-four pairs of male rats and the twenty quadruple groups of female rats and for body lengths in fifteen of the latter twenty groups fail to show any superiority for either of these sugars, when fed in rations in which they were substituted for starch at a level of 25% of the diet. One may conclude, therefore, that when fed at moderate levels lactose is equivalent to sucrose in growth-promoting value and that both are comparable to starch.

Conclusions. In the studies in which a system of control feeding was employed it was found that lactose had no greater power than sucrose to stimulate increase in weight or length, and that neither had exhibited, at the levels fed, any growth promoting value in addition to that given by the polysaccharide starch. With these findings in mind, one would hesitate to interpret the results of the ad libitum series as indicating a special growth promoting property of lactose. Although in series 1 galactose was not observed to stimulate gains in weight, a statement as to its growth promoting value should be withheld until it has been fed under conditions of control feeding.

ASH CONTENT OF BONES

As influenced by sucrose. A comparison of sucrose with starch was made in series 2 (table 2). Of the twenty-one pairs of rats there were five in which both members had approximately the same amount of bone ash, eight which favored the starch ration, and eight which favored the sucrose ration. No differences could be found on statistical analysis of these data (odds of 2 to 1) or in the averages for the two groups. Therefore, the substitution of 25% sucrose for an equivalent portion of starch had conferred no additional calcifying properties to the ration.

As influenced by lactose. In the two series in which lactose was compared with starch, the disaccharide was found to have the greater calcifying power. For series 1 individual litter response to the three diets are given in figure 1. Litter variation is seen to be great. Yet, in all but two cases, the animal

TABLE 2

Gains in weight and final body lengths for the twenty groups of animals in series 2

LITTER MATE GROUPS	FOOD INTAKE (28 DAYS)	GAIN IN WEIGHT (28 DAYS)				FINAL BODY LENGTH			
		Starch ration	Cod liver oil ration	Lactose ration	Sucrose ration	Starch ration	Cod liver oil ration	Lactose ration	Sucrose ration
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*mm.*	*mm.*	*mm.*	*mm.*
3956–59	160	31	41	38	32	152	152	150	152
3970–73	158	38	50	37	43	156	163	160	156
3981–87	192	37[1]	46	42[1]	36[1]	158	162	155	155[1]
3988–91	115	12	24	13	12	....	....	....	....
4001–05	142	28[1]	34	28	29	154	153	148	145
4006–12	152	28[1]	52	39[1]	37[1]	....	....	....	....
4016–21	152	34	42	32[1]	27[1]	154	157	151[1]	149[1]
4029–34	151	32	41	36[1]	38[1]	146	155	156	156
4045–48	179	36	49	43	43	....	....	....	....
4203–09	198	51	54	43	47	158	162	158	162
4205–10	198	53	56	43	53	160	158	153	159
4278–85	117	25	26[1]	24[1]	26[1]	....	....	....	....
4411–14	181	40	42	35	43	160	159	157	157
4415–18	155	26	30	24	29	152	156	150	157
4448–53	163	36	40	34	44	....	....	....	....
4517–20	121	18	14	9	12	140	145	141	143
4726–29	193	42	48	44	46	166	166	165	160
4730–33	148	28	29	29	29	153	155	150	152
4734–37	186	45	42	32	38	157	160	156	156
4738–41	137	23	25	29	30	153	159	153	151
Average	160	33	39	33	35	155	158	154	154
Standard deviation	25.6	10.0	11.2	9.5	10.6	5.9	5.0	5.5	5.2
Probable error	17.3	6.7	7.6	6.4	7.1	4.0	3.4	3.7	3.5

[1] Average for two animals.

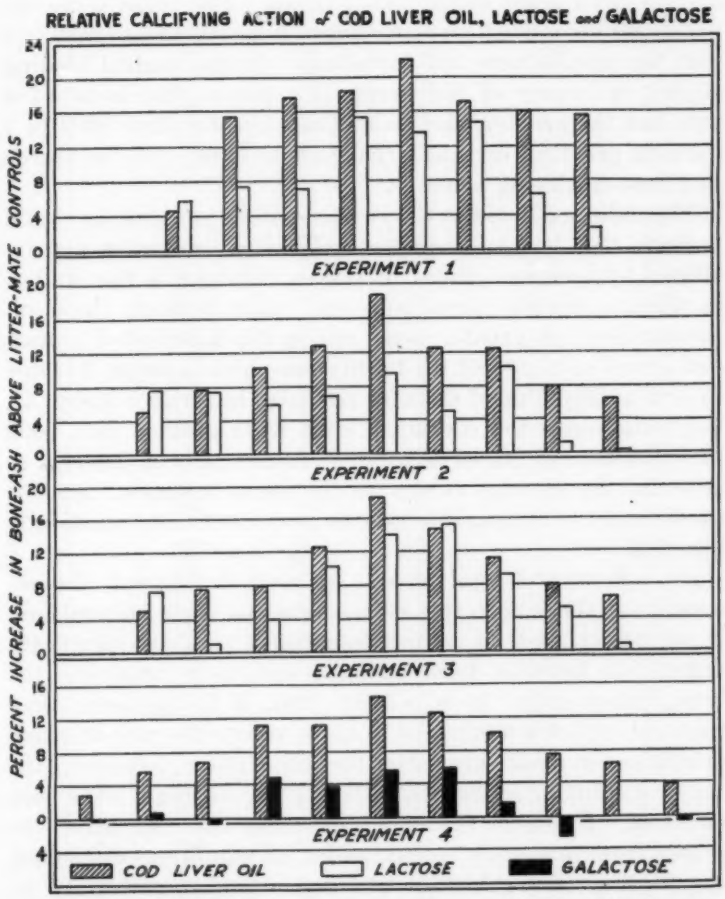

Fig. 1 The calcifying action of lactose, galactose and cod liver oil in relation to the basal ration is graphically presented here for each litter mate group in series 1. In plotting the data the relative amount of ash in the bones of the rat on the basal food was placed at 0, forming a base line. The increase or decrease from this value, observed for the other two rats of the litter mate groups, is represented in columns above or below this line in terms of per cent.

receiving lactose had significantly more ash in its bones than had its litter mate on the basal ration. For these three experiments the statistical odds ranged from 999 to 1 to 2499 to 1 that the results were not fortuitous. In the control feeding studies in twenty of the twenty-one groups, the lactose-fed rats had the greater bone ash. Odds greater than 9999 to 1 indicate practical certainty that lactose is superior to starch as a bone-calcifying agent.

The data presented in the two preceding paragraphs would indicate that lactose possesses calcifying properties not exhibited by sucrose. Proof of this is obtained in the studies in which a direct comparison was made between these disaccharides. A greater bone ash in the lactose-fed animal was found in twenty of the twenty-one pairs in series 2 (table 4) and in fifty-four of the fifty-six pairs in series 3 (table 3). For both series the statistical odds were greater than 9999 to 1 that the results were not accidental. It is of interest to note that the average values for the males in series 3 and for the females on comparable rations in series 2 were practically identical.

As influenced by galactose. As seen in figure 1, the responses of the rats to this monosaccharide were inconsistent. A statistical analysis of the data failed to show significant differences between these animals and those receiving the basal ration only. It is possible that, if the galactose had been fed in such a manner as to approximate the rate at which galactose is made available for absorption following the enzymic splitting of lactose, different results might have been obtained. Probably the only conclusion that can safely be drawn from this study is that, under the conditions employed, the feeding of d-galactose was not attended uniformly by increased deposition of ash into the skeleton, whereas comparable amounts of galactose fed in the form of lactose induced more normal calcification.

As influenced by cod liver oil. In general the highest values for bone-ash found in this study were in the rats receiving cod liver oil (see fig. 1 and tables 1 and 4). In six of the

TABLE 3

Individual responses in appetite, growth and calcification of fifty-six pairs of males studied in series 3

LITTER MATE PAIR NO.	FOOD INTAKE (35 DAYS)	GAIN IN WEIGHT (35 DAYS)		ASH CONTENT OF BONES[1]	
		Lactose ration	Sucrose ration	Lactose ration	Sucrose ration
	gm.	*gm.*	*gm.*	%	%
1	198	55	65	57.7	51.1
2	225	60	62	56.5	52.7
3	241	87	73	56.8	54.8
4	192	58	66	54.6	51.5
5	221	74	75	55.3	51.5
6	218	60	60	54.7	54.3
7	194	38	46	57.1	52.9
8	215	60	62	57.0	55.8
9	196	50	59	55.8	52.9
10	227	63	65	56.0	55.0
11	248	77	72	56.7	55.1
12	225	63	76	57.9	56.9
13	226	76	60	57.5	54.2
14	164	37	43	56.1	54.4
15	251	60	71	54.8	50.7
16	236	62	69	57.1	53.1
17	261	82	84	57.7	52.5
18	238	71	71	58.9	53.9
19	238	74	74	55.6	53.6
20	234	77	75	55.5	53.6
21	231	74	73	56.1	52.7
22	310	90	88	58.6	55.0
23	235	68	71	56.5	51.9
24	264	83	81	57.9	53.6
25	214	41	47	56.8	52.4
26	261	81	84	57.6	54.4
27	240	63	84	56.2	54.4
28	296	92	91	58.7	56.3
29	244	79	76	57.8	52.0
30	306	91	104	59.5	54.8
31	373	121	106	59.1	56.6
32	221	74	74	57.1	52.0
33	215	78	73	58.1	57.5
34	241	80	80	58.4	58.3
35	209	64	70	55.1	54.6
36	246	78	77	56.3	53.8
37	262	84	79	58.3	52.6
38	201	68	63	56.2	52.7
39	214	74	72	54.7	52.6
40	208	80	63	56.4	53.7
41	188	59	62	56.8	53.1
42	200	68	72	57.1	53.3
43	185	60	66	55.3	50.3
44	165	49	55	52.2	51.0
45	175	69	54	54.4	48.5
46	161	47	49	54.3	45.5
47	134	40	42	52.8	48.6
48	195	56	57	55.5	55.0
49	170	61	60	54.0	51.2
50	166	58	56	55.2	55.7
51	150	52	55	55.7	51.8
52	143	46	45	54.5	52.7
53	149	43	48	52.8	48.8
54	157	50	52	56.3	53.9
55	153	....	....	57.9	51.2
56	144	....	....	53.5	48.9
Average	216	67	68	56.3	53.1
Standard deviation	45.8	16.0	13.9	1.6	2.3
Probable error	30.9	10.8	9.4	1.1	1.6

[1] Calculated on basis of dried, alcohol-ether extracted bone.

twenty-one cases in the control feeding series the lactose-fed rat had a higher bone-ash content than those receiving the above supplement, yet the statistical odds were 140 to 1 that cod liver oil was the better calcifying agent.

TABLE 4

Ash content of bones of rats in series 2

LITTER MATE GROUPS	FOOD INTAKE (28 DAYS)	ASH CONTENT OF BONES[1]			
		Starch ration	Cod liver oil ration	Lactose ration	Sucrose ration
	gm.	%	%	%	%
3956–59	160	53.8	58.6	58.4	56.6
3970–73	158	55.9	58.5	57.9	56.4
3981–87	192	55.8[2]	58.5	59.7[2]	55.4[2]
3988–91	115	54.0	57.6	54.5	51.3
4001–05	142	51.8[2]	55.8	55.6	52.6
4006–12	152	53.1[2]	58.0	55.8[2]	54.7[2]
4016–21	152	55.8	57.2	56.5[2]	52.2[2]
4029–34	151	50.1	55.5	55.7[2]	54.5[2]
4045–43	179	53.1	58.0	56.3	52.2
4203–09[3]	198	56.4	57.9	56.6	56.6
4205–10[3]	198	55.4	56.8	57.4	53.9
4278–85[3]	118	49.7	55.6	53.6	49.6
4279–81[3]	117	50.7	54.4	53.6	50.6
4411–14	181	55.7	58.4	55.7	52.8
4415–18	155	53.0	57.6	58.5	54.0
4448–53	165	53.2	57.0	58.4	52.4
4517–20	121	51.2	53.7	53.7	49.4
4726–29	193	56.1	58.0	58.2	53.2
4730–33	148	53.6	58.9	58.1	53.5
4734–37	186	53.6	60.0	55.9	55.1
4738–41	137	55.6	58.7	57.1	55.9
Average	153	53.7	57.4	56.5	53.5
Standard deviation	26.6	2.0	1.5	1.7	2.1
Probable error	17.9	1.3	1.0	1.1	1.4

[1] Calculated on basis of dried, alcohol-ether extracted bone.

[2] Average for two animals.

[3] Litter mate groups.

The addition of vitamin D in series 2 did not produce bones of maximal ash content (except in two cases). The values, however, are consistent with data reported earlier for rats receiving restricted food and cod liver oil (Outhouse, Smith and Merritt, '37).

Comparison of these data with previously published work. This calcifying activity of milk sugar has been noted previously. Thus, Jarvis ('30) had reported lactose to be superior to sucrose for rats; and Kline, Keenan, Elvehjem and Hart ('32) had found that, in contrast to maltose or citric acid, it induced chicks on rachitogenic ration to deposit a greater amount of ash in their skeletons. Whittier, Cary and Ellis ('35) have presented data secured under conditions of control feeding. In three of four pairs of rats, fed approximately 4 months, the lactose-fed rat had a greater body-ash content than the sucrose-fed rat. In three pairs of pigs, the ones receiving lactose showed a higher ash content in the edible portion of their carcasses than was found in the sucrose-fed pair mates. This is an indication that the soft tissue as well as the skeleton may be influenced by the inclusion of moderate amounts of lactose in the diet. Possible proof of this is found in the work of Mitchell and Dodge ('35). In their studies on high levels of lactose ash was deposited in the eye to the extent that the calcium content was approximately twice as great as that found in control animals. Evidences of cataractous eyes were not found in the animals during the short period of these studies herein reported.

Conclusions. The results obtained in these studies demonstrate that, when lactose comprised 23 to 26% of the caloric intake of rats, better calcified bones resulted than when comparable amounts of sucrose were given or when starch was the only carbohydrate fed. Galactose in amounts equivalent to that found in these levels of lactose caused no increase in bone ash. Inasmuch as the mineral intake was controlled in each of the two technics employed, it is felt that the observed variations in bone ash induced by these four carbohydrates must be due to differences in their physiological values.

SUMMARY

Under conditions of controlled mineral intake lactose, sucrose, and galactose were fed to young litter mate rats in amounts equivalent to 23 to 26% of their caloric intake. With ad libitum feeding the supplementation of the basal diet with lactose induced greater gains in rats than were obtained in litter mates on the basal diet, but only when the former consumed appreciably more calories. On paired feeding no alteration in growth was observed when 25% of sucrose or lactose replaced an equivalent amount of starch in the basal ration. Of these carbohydrates lactose was the only one which caused an acceleration in bone calcification. This effect was found under both ad libitum and control feeding conditions. In comparison with cod liver oil, however, this sugar, at the levels fed, did not induce as well calcified bones.

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THE NUTRITIVE VALUE OF THE PROTEINS OF NUTS IN COMPARISON WITH THE NUTRITIVE VALUE OF BEEF PROTEINS

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It is a singular fact that in human nutrition diets are universally planned on the basis of the chemical composition of available foods, while in animal nutrition the balancing of rations is commonly undertaken on the basis of the contents of digestible nutrients in animal feeds. The observation that human foods are ordinarily more digestible than animal feeds is only a partial justification for this difference since many vegetables and fruits are relatively indigestible. The digestibility of human foods has not been systematically studied, although many scattered determinations may be found in the literature. The practical value of such work is obvious.

Nutrients undergo wastage in the body not only in the gastrointestinal tract, but also in metabolism. The metabolic wastage of protein is conditioned to a large extent by its content of those amino acids indispensable for animal life, and to some extent by the protein content of the diet fed. A metabolic wastage of all organic nutrients is represented in the specific dynamic effect of a meal. Clearly, the complete evaluation of a food or a diet as a source of nutrients to the animal must consider all manner of wastage in the course of its assimilation, since any food is of value to the body, not in proportion to what the food contains, but in proportion to what the body can extract from it for its own uses. This conception of the

'net value' of a food, as contrasted with its 'gross value' measured by a chemical analysis, is the contribution of H. P. Armsby, the significance of which seems to be appreciated only in animal nutrition.

The term 'biological value' of a protein was first introduced by Thomas to designate that percentage of the absorbed protein that is retained in the body for its own uses. Hence the magnitude of this value will vary inversely with the wastage of protein in metabolism. The digestion coefficient and the biological value of the protein fraction of a food would seem, therefore, to define completely its nutritive value, and, in combination with the percentage of protein in the food, to define what has been called the 'net protein content' of the food (Mitchell, '27).

Quantitative studies of the nutritive value of foods are among the most practical contributions of the science of nutrition. In particular, they facilitate the nutritional classification of foods, and a better understanding of their nutritional equivalence. For example, on the basis mainly of their high contents of protein and of fat, meat and the oil-bearing nuts have been considered equivalent in nutrition; in other words, nuts have been proposed as adequate meat substitutes. However, this nutritive equivalence may be more apparent than real, since it is based almost entirely upon chemical composition.

It will be the purpose of this study to assess quantitatively the protein value in nutrition of nuts as compared with meat (beef); in this paper, results obtained on the almond, the Brazil nut, the filbert, the cashew nut, and the English walnut will be reported. These five nuts with the peanut and the pecan previously studied (Mitchell, Burroughs and Beadles, '36) are among the most popular varieties consumed in this country.

The digestibility of the protein of nuts has not been very thoroughly or extensively studied. Holmes ('18) has reported that the protein of peanut flour is digested by human subjects to the extent of 86%, while Cajori ('18), in experiments on

both men and dogs, obtained coefficients of digestibility of 80 to 92% for the protein of diets containing unreported amounts of nuts. On the other hand, Jaffa ('03) has observed among the fruitarians of California a much lower digestibility of the protein of rations containing large amounts of nuts; for rations containing protein derived largely from walnuts (63 to 86%) eight coefficients averaging 74.4 were obtained; in two cases, rations containing large proportions of peanut protein were digested to the extent of 87 and 78%, but in five digestion trials with rations containing pecans, furnishing generally about half of the total protein intake, an average coefficient of only 70 was obtained. Jones and Waterman ('22) have reported a low digestibility of arachin, the main protein of the peanut, in enzyme digestion experiments. A variable digestibility of nut protein is to be expected according to Heupke ('31) depending upon the completeness of mastication; an extremely low digestibility may follow poor mastication. We have obtained (Mitchell, Burroughs and Beadles, '36) coefficients of true digestibility with rats of 71 for pecan proteins and 96 for peanut proteins.

A number of feeding experiments to test the nutritive value of nut proteins, either alone or in combination with other food proteins, have been published. Sure ('20) was unable to secure good growth in rats on rations containing as high as 18% of arachin or of the mixture of arachin and conarachin extracted from peanut meal by salt solutions. Using the numerical method of Osborne, Mendel and Ferry to express the nutritive value of dietary protein (gain per gram of protein consumed), Maynard, Fronda and Chen ('23) obtained higher values for peanut oil meal (average of 1.45) than for corn meal (average of 1.18) at a protein level of 9%, together with some indication of a moderate supplementary relation between the proteins of these two foods. Shiba and Koyama ('23) also obtained indications of a high nutritive value of peanut protein fed at a 14% level, while Pian ('30) in nitrogen balance studies obtained a biological value of only 59 for the proteins of the Chinese peanut with a diet containing 10% of

protein. In the previous study (Mitchell, Burroughs and Beadles, '36) from this laboratory on the nutritive value of nuts, biological values of 58 for the peanut and 60 for the pecan were obtained with rations containing 7.6% of digestible protein calories, using rats as subjects. These investigations are somewhat difficult to reconcile.

The supplementary relations between the protein fraction of any high protein food and of wheat flour is of great practical significance in dietetics, because wheat endosperm proteins themselves possess such a low biological value. Johns and Finks ('20) found that a bread containing 25 parts of peanut flour and 75 parts of wheat flour furnished adequate proteins and water-soluble vitamins for 'normal' growth when fed at a 10% protein level. Eddy and Eckman ('23) have confirmed the high nutritive value of a mixture of peanut and wheat flour and have compared this flour with a mixture of wheat flour and meat residue, also containing 6 to 7 parts of wheat flour protein to 10 parts of total protein. The peanut flour mixture promoted somewhat better growth and markedly better reproduction than the meat residue mixture at the same level of protein intake. This superiority of the peanut flour mixture is attributed by the authors of the report to a superior supplementing value of the peanut proteins, although they admit that the two flour mixtures were the sole source of vitamin B (the complex) as well as of protein. The meat residue was prepared in such a way that the major part of the contained water-soluble vitamins would be removed.

In unpublished experiments we have shown that a mixture of peanut flour and patent white flour in the proportion of 1 to 3, the nitrogen of which was derived from the two flours to the extent of 62 and 38%, respectively, was somewhat higher in biological value for young albino rats than either flour alone, but was much lower in biological value than a similar mixture of beef and white flour proteins, contrary to the above cited conclusion. The respective biological values, determined with ten rats in each test, were 59.6 and 80.5. The expected values if no supplementary relations existed are

54.3 and 67.9, respectively. The marked superiority of the proteins of the beef-white flour mixture over those of the peanut-white flour mixture was confirmed by a paired-feeding test (Mitchell and Beadles, '30).

Concerning the English walnut and the pecan, little work has been reported with reference to their values as sources of dietary protein. Cajori has obtained good growth in rats with walnut proteins ('20), and pecan proteins ('21) when fed at a concentration of 18%, but such qualitative data indicate merely that the proteins are complete in their amino acid constitution and are adequate for growth. The nutritive value of walnut proteins for rats and mice was more extensively studied by Mignon ('23), who obtained 'normal' growth with the total proteins of the walnut at a 12% level of protein, and about the same results with walnut globulin. The residual proteins after extraction of the globulins induced only an unsatisfactory growth even at an 18% level of intake. The gain per gram of protein consumed was not particularly high in any of these tests, never exceeding 1.39 gm. for rats or 0.50 gm. for mice.

In the investigations of nut proteins reported below it was expected that the most significant determinations of nutritive value would be obtained only by the use of quantitative methods involving the separation of the wastages of nitrogen in digestion and in metabolism. The biological values of the various protein mixtures were determined according to the most improved method developed in this laboratory (Mitchell, Burroughs and Beadles, '36).

PLAN OF EXPERIMENTS

The nutritive values of the proteins of the almond, English walnut, Brazil nut, cashew nut and filbert were studied by determining the nitrogen intake and outgo of the rats under conditions standardized so that significant biological values could be estimated (Mitchell, '24; Mitchell and Carman, '26; Mitchell, Burroughs and Beadles, '36). The rations used throughout were adequate for good growth in all known respects except for the protein mixture under study. They

contained 22% of fat, instead of the usual 10 to 12%, in the hope that the consistency would be such as to minimize scattering. Since this hope was not realized, all food portions after being weighed into the food cups were mixed with water to a thin consistency, an expedient which had the desired effect. In previous work, a level of 8% of dietary protein has been adopted, but with the higher fat content of the diets, the protein content was raised from 8 to approximately 9.2% in order to maintain constant the percentage of protein calories in the diet at 7.6%.

The biological values of the dietary proteins were determined upon groups of four to twelve growing rats, the initial weights of which varied from 50 to 120 gm. The plan of the experiments varied, from the simplest type, such as one in which a filbert ration was fed in period 1, an almond ration in period 3, and the standardizing ration containing 4% of egg protein in the intermediate period, to one extending over five or six periods in which three nuts were compared, with intervening standardizing periods. The Brazil nut, the filbert, and the cashew nut were compared directly with beef by feeding half of the group a nut ration and the other half a beef ration; then, after a standardizing period, the feeding was reversed. In such experiments as the latter, paired-feeding was used in order to obviate any possibility of error due to a variable intake of food.

The low-nitrogen ration used, containing 4% of whole egg protein, was the standardizing ration, upon which were determined the metabolic nitrogen of the feces per gram of dry matter consumed and the endogenous nitrogen per 100 gm. of body weight. These values were applied without adjustment to the preceding and following periods in those tests covering only three periods. The collection periods were of 7 days duration, with transition periods of at least 4 days.

The nuts to be tested were analyzed in the routine way, with the results given in table 1. The results previously reported for the peanut and the pecan (Mitchell, Burroughs and Beadles, '36) are included for comparison. The gross energy

value was determined with the bomb calorimeter. Before incorporation into the experimental rations, the nuts were finely ground, dried before a blower at temperatures not exceeding 50°C., extracted repeatedly (but not exhaustively) with ether, dried and again analyzed for nitrogen and fat. The beef round, containing the protein with which the nut proteins were compared, was trimmed of visible fat, dried before the blower, ground finely, extracted with ether, dried again and also analyzed for nitrogen and fat.

TABLE 1

The chemical composition of the nuts tested

DESCRIPTION	MOISTURE	CRUDE PROTEIN	ETHER EXTRACT	CRUDE FIBER	NITROGEN-FREE EXTRACT	ASH	CALCIUM	PHOSPHORUS	GROSS ENERGY IN CALORIES PER GRAM
	%	%	%	%	%	%	%	%	
Brazil nuts, fresh	3.50	16.25	69.75	4.48	2.85	3.17	0.195	0.664	7.55
Cashew nuts, fresh	4.39	19.52	48.70	1.20	23.72	2.47	0.041	0.507	6.48
Almonds, blanched	4.56	21.94	56.75	3.34	10.29	3.12	0.251	0.527	6.76
Filberts, fresh	3.70	13.50	70.14	5.26	5.55	1.85	0.100	0.305	7.57
Pecans, fresh	4.30	11.28	72.96	4.90	5.02	1.54	0.073	0.315	7.69
English walnuts, fresh	4.39	21.16	61.91	2.35	8.07	2.12	0.096	0.463	7.32
Peanuts, raw, without skins	5.24	28.25	51.26	1.89	10.84	2.52	0.049	0.553	6.85

The analyses of pecan nuts are the averages for three samples obtained from as many consignments. For Brazil nuts, two consignments are represented. The analyses of other nuts relate to one sample from one consignment.

Of the experimental rations, it may be said of all that they contained approximately 9.2% of protein, 22% of fat, 5% of the Wesson modified Osborne and Mendel salt mixture (Wesson, '32), 1% of NaCl, and 10% of sucrose. The fat was provided by the test sample, 8% of butter fat, 2% of cod liver oil, and lard in an amount needed to make up 22% of total fat. Vitamins B and G were provided by 1% of dried yeast and Harris vitamin powder given in separate small dishes at the rate of 5 mg. per gram of food; in most tests, however, these vitamins were supplied by giving each rat daily 3 to 5 drops of a solution containing for each 100 cc.

20 gm. of Harris vitamin pow. and 20 gm. of sucrose. At times the dosage of yeast vitamin was increased in attempts (not always successful) to restore a failing appetite. All rations were completed by variable additions of corn starch. The gross energy content approximated 4.8 cal. per gram.

THE RESULTS OBTAINED

It seems unnecessary to give the nitrogen metabolism data and all of the calculations leading to the coefficients of true digestibility and the biological values obtained in these numerous experiments, since the method of calculation has been explained in previous publications from this laboratory. In all experimental periods the rats gained in weight, albeit the gain was quite variable. In comparative tests the attempt was made to equalize the intake of food, and in most cases this ideal was realized. The average coefficients of true digestibility and the average biological values, with their probable errors computed according to the classical method, are summarized in table 2. Again the results for the peanut and the pecan, previously reported (Mitchell, Burroughs and Beadles, '36), are included for comparison.

In digestibility and in biological value, the proteins of beef round are superior to those of any of the nuts studied; generally the difference in biological value is great, representing an improvement over the nut proteins of 25 to 50%. The difference in digestibility is not great, when beef proteins are compared with the proteins of the peanut, the Brazil nut, the cashew nut or the almond. However, the proteins of the pecan are relatively indigestible, 71%, while those of the English walnut are only 84% digestible. These facts should be considered in making any comparison of the nutritive value of meats and nuts, for example, the statement sometimes made that nuts are 'meat substitutes.'

While the biological values of all other nuts under the conditions of these experiments fall within the range 50 to 60, the cashew nut is a prominent exception, possessing a value of 72, only slightly below that of beef round, 76.

The filbert rations afforded us the first prominent instance of the circumstance noted occasionally (Kon, '28; Turk, Morrison and Maynard, '35) by others, namely, that biological values determined immediately after a period of low-nitrogen feeding tend to be higher than those obtained after periods of test-protein feeding. This has been interpreted (Chick, Hutchinson and Jackson, '35) as the result of a 'nitrogen debt' incurred during specific protein starvation. However, another possibility more in harmony with the very occasional appearance of this phenomenon (Mitchell, '28) is that in the preceding low-nitrogen feeding period the endogenous level of nitrogen excretion had not been attained, so that the biological values of the following period more than those of subsequent periods, are fictitiously high.

TABLE 2

The true digestibility and the biological value of nut proteins and of beef proteins

SOURCE OF PROTEIN	NUMBER OF TESTS	TRUE DIGESTIBILITY		BIOLOGICAL VALUE	
		Average and probable error [1]	Standard deviation [2]	Average and probable error [1]	Standard deviation [2]
Beef round	41	100	...	75.78 ± 0.41	3.86
Brazil nut	14	95.71 ± 0.22	1.24	54.07 ± 0.45	2.52
Cashew nut	22	96.23 ± 0.16	1.11	72.50 ± 0.66	4.56
Almond	19	93.95 ± 0.23	1.50	50.84 ± 0.37	2.37
Filbert	14	91.29 ± 0.20	1.09	50.29 ± 0.67	3.75
Pecan	10	70.71 ± 0.45	2.13	59.80 ± 0.50	2.32
English walnut	9	84.11 ± 0.22	1.00	55.89 ± 0.92	4.11
Peanut, raw	10	97.39 ± 0.27	1.27	57.9 ± 1.1	5.30
Peanut, roasted	10	96.15 ± 0.22	1.05	55.80 ± 0.77	3.59

[1] These probable errors do not measure reliably the significance of differences between averages for two different sources of protein when both have been tested with the same group of rats. In such cases, when the two results obtained with the same rat are considered as paired observations, and 'Student's' method of analysis applied, the difference between averages assumes a greater significance. Thus, judged by the probable errors in the above table, the difference in biological value between the proteins of raw and roasted peanuts is not significant, but when the average difference is considered as the average of a series of paired differences using 'Student's' method of analysis, it becomes highly significant (Mitchell, Burroughs and Beadles, '36).

[2] Standard deviations computed by the formula $s = \sqrt{\frac{\Sigma\delta^2}{n}}$

With the filbert, fourteen determinations of biological value made before a standardizing period averaged 50, while eleven determinations carried out immediately following a standardizing period averaged 63.

In determining which of these two values is the true one, the filbert was compared with the cashew nut (for which, all biological values were consistent) in a modified paired-feeding test in which paired rats are given the same amount of food and the level of the better protein under comparison is lowered until equal gains are secured. This method is explained in a previous publication from this laboratory (Mitchell, Burroughs and Beadles, '36). In eleven pairs of rats carried along in this way for 9 weeks (except for one pair discontinued at the end of 38 days), on the average, 1 gm. of digestible nitrogen from the cashew nut promoted the same rate of growth as 1.41 gm. of digestible nitrogen from the filbert. Analysis of the rats at the termination of the feeding period revealed no significant difference in the storage of nitrogen between pair mates.

Taking the biological value of cashew nut protein as 72, that of the filbert may be computed from the proportion $72 : x :: 1.41 : 1$. The value of $x = 51$, approximately the same as the average of 50 obtained by the nitrogen metabolism method before a standardizing period. Therefore, this value is taken in preference to the higher value of 63, obtained after low-nitrogen feeding.

Referring to table 2 again, it will be noted that the standard deviations of the individually determined biological values range from 2.32 to 5.30, averaging 3.60. This is practically the same as the average precision previously noted (Mitchell, Burroughs and Beadles, '36) in this determination as carried out on growing rats by the method perfected in this laboratory, measured by an average standard deviation of 3.7.

SUMMARY AND CONCLUSIONS

The true digestibility and the biological value of the proteins of beef round and of five nuts, Brazil, cashew, almond, filbert and English walnut, have been determined in from nine to forty-one tests in each case, using growing albino rats as subjects.

Beef protein is superior to all of the nut proteins studied in both particulars, although the cashew nut is not greatly inferior, with a digestibility of 96 and a biological value of 72. All other nuts exhibit biological values under the conditions of these experiments ranging from 50 to 60.

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NUTRITIONAL WELL-BEING AND LENGTH OF LIFE AS INFLUENCED BY DIFFERENT ENRICHMENTS OF AN ALREADY ADEQUATE DIET

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ONE FIGURE

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The possibility of improving an already adequate diet, with resulting enhancement of nutritional well-being and increase in length of life, has been shown in previous papers (Sherman and Campbell, '24, '28, '30, '35 b).

Using rats as experimental animals, in a study of the proportion of protective food needed to make adequate a food supply otherwise composed of cereal grain, table salt, and distilled water, we found that 1 part of dried whole milk to 5 parts of ground whole wheat (our diet A) was adequate; but that a mixture of one-third whole milk powder with two-thirds ground whole wheat (our diet B) gave better results as judged by observations upon growth, adult vitality, and length of life.

Striking evidence that diet A is adequate, in the full sense in which the word is understood in nutritional research and in human medicine, is afforded by the fact that it supports normal growth and health, reproduction and length of life, generation after generation. (Families are still thriving in the fortieth generation on this diet at the time of writing.) Diet B differed from diet A in the sole experimental variable that it contained a larger proportion of milk. In chemical terms, however, this change involved enrichment in more than

one factor; most markedly in calcium, vitamin A, and the riboflavin factor which in previous papers from this laboratory has been called vitamin G. The new experiments here described were designed to ascertain the factors or fractions of milk which were actually concerned in the improvements of nutritional well-being and length of life which had resulted from the increased proportion of milk in the diet. Careful consideration of the existing state of knowledge led us to plan these experiments partly in terms of the element calcium and partly in terms of the natural fat-soluble and water-soluble fractions of milk. For it was deemed wiser to supply the vitamins A and G (riboflavin) accompanied by their natural concomitants than in purified forms which, in addition to the question of strict chemical purity, would involve (from the viewpoint of this particular investigation) an assumption that no other known or unknown substance could have been a significant factor in the nutritional difference between diets A and B.

EXPERIMENTAL

Diets. Five diets were used, the basis of each being ground whole wheat and dried whole milk: 1) diet A, known in the laboratory as diet 16; 2) to this was added sufficient calcium carbonate to give the same calcium content as in diet B (diet 162); 3) or sufficient butterfat to give the same content of milk fat as in diet B (diet 165); 4) or both calcium carbonate and butterfat in same amounts as in diets 162 and 165 (diet 166); 5) or skim milk powder replacing one-fifth of the wheat, thus enriching the diet in milk proteins, calcium, and riboflavin or vitamin G, and possibly other factors not yet fully known (diet 167). The composition of the diets is given in table 1.

Experimental procedure. Parallel lots of rats, each lot consisting of two males and three females, were made up when the rats were 4 weeks of age, the members in each lot being matched as to weight, sex, and heredity with those in the other lots formed at the same time. All rats used were from families that had been on diet A (diet 16) for twenty generations or more. In this fivefold comparison it was not possible

to place lots of litter mate parallels on every diet each time; but, whenever possible, three such lots were started in parallel, and these were systematically distributed among the diets in rotation so that there were the same number of lots on each diet and the same final parallelism between all diets.

For each of the diets here compared there were twelve fresh starts with lots in direct litter mate comparison with parallel starts on others of the diets, the animals of each such fresh start being known as the 'first generation' on their respective diets except in the case of diet 16 (diet A) which as above explained was the stock diet from which all initial animals were drawn.

TABLE 1

Percentage composition of diets

	DIET 16	DIET 162	DIET 165	DIET 166	DIET 167
Ground whole wheat	81.97	81.66	77.44	77.14	65.79
Whole milk powder	16.39	16.33	16.41	16.35	16.45
Calcium carbonate	.....	0.39	.....	0.38	.....
Butterfat	.....	.....	4.59	4.58	.....
Skim milk powder	.....	.....	.....	.....	16.45
Sodium chloride	1.64	1.63	1.56	1.55	1.31
Calories per gram	3.73	3.71	3.98	3.97	3.75
Protein	13.1	13.0	12.6	12.5	16.8
Calcium	0.195	0.337	0.184	0.336	0.367

These initial or first generation animals constituted about one-third of the total numbers included in this comparison, the other two-thirds consisting of their offspring, representatives of which were continued on their parents' diet and were grouped when 4 weeks of age into breeding lots of two males and three females each. Guided by our previous experience as to the number of generations which may be required to show the full effects of relatively small differences of diet, we included in the present comparison representatives of the second, third and fourth generations on diets 162, 165, 166 and 167. These were selected 1) at different seasons of the year, that there might be no unbalanced seasonal influence, 2) so that advanced generations on each of the diets would

be about parallel, and 3) from litters of average weight for the diet.

All experimental animals, either in the first or subsequent generations, were kept until death occurred from natural

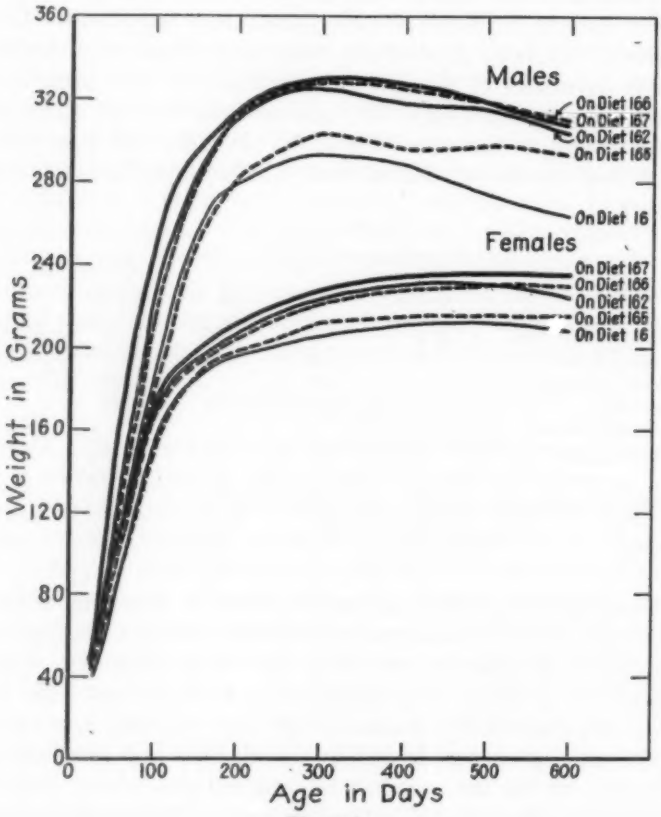

Figure 1

causes, except in some cases where larger numbers were kept for early growth records. The numbers on each diet were from 66 to 77 males, 101 to 111 females, the exact number on each diet being given in table 2, together with the quantitative results of the experiments.

TABLE 2

Comparison of data of experimental animals

DESIGNATION OF DIET	DIET 16	DIET 162	DIET 165	DIET 166	DIET 167
NATURE OF ENRICHMENT	Our basal diet A (no enrichment)	Calcium (as carbonate)	Butterfat (vitamin A)	Calcium and butterfat	Riboflavin, Ca, and protein[1]
Growth from twenty-eighth to fifty-sixth day of age					
Males					
(Number of cases)	(73)	(83)	(66)	(80)	(77)
Gain in grams ± P.E.	56.3 ± 1.05	71.0 ± 0.87	51.0 ± 1.00	66.1 ± 0.86	96.4 ± 1.23
Coefficient of variation	23.9	16.7	24.0	17.4	16.7
Females					
(Number of cases)	(107)	(116)	(102)	(114)	(112)
Gain in grams ± P.E.	47.0 ± 0.69	60.2 ± 0.63	45.2 ± 0.64	57.9 ± 0.52	73.5 ± 0.61
Coefficient of variation	22.8	16.9	21.4	14.4	13.1
Gain per 1000 Calories in lots of two males and three females					
(Number of cases)	(35)	(38)	(36)	(36)	(37)
Grams ± P.E.	64.7 ± 0.94	71.7 ± 0.90	57.8 ± 0.72	66.1 ± 0.81	89.8 ± 1.02
Coefficient of variation	12.9	11.6	11.2	11.1	10.4
Gain per gram of protein in lots of two males and three females					
(Number of cases)	(35)	(38)	(36)	(36)	(37)
Grams ± P.E.	1.83 ± 0.027	2.04 ± 0.026	1.83 ± 0.023	2.02 ± 0.024	2.00 ± 0.023
Coefficient of variation	12.9	11.7	11.2	10.5	10.4
Age at birth of first young					
(Number of cases)	(90)	(98)	(96)	(104)	100
Days ± P.E.	132 ± 2.4	111 ± 1.6	134 ± 2.4	107 ± 1.3	110 ± 2.1
Coefficient of variation	26.1	21.4	26.1	18.3	28.3
Duration of capacity to reproduce					
(Number of cases)	(101)	(102)	(102)	(111)	(109)
Days ± P.E.	213 ± 9.4	279 ± 10.2	286 ± 10.1	241 ± 9.6	234 ± 10.0
Coefficient of variation	66.9	55.3	53.4	63.1	67.0
Young born					
(Number of cases)[2]	(101)	(102)	(102)	(111)	(109)
Young per female ± P.E.	24.1 ± 1.13	32.6 ± 1.34	34.2 ± 1.33	27.7 ± 1.27	27.6 ± 1.26
Coefficient of variation	70.6	62.1	58.9	72.7	71.3
Young reared					
(Number of cases)[2]	(101)	(102)	(102)	(111)	(109)
Young per female ± P.E.	12.7 ± 0.74	20.4 ± 1.12	19.0 ± 0.96	15.3 ± 0.89	16.1 ± 0.94
Coefficient of variation	88.1	83.3	76.4	92.6	91.0
Success in rearing young					
(Number of cases)[3]	(90)	(98)	(96)	(104)	(100)
Per cent young reared	46.0 ± 2.1	56.6 ± 2.0	54.2 ± 1.9	50.8 ± 2.1	53.1 ± 1.9
Coefficient of variation	66.2	52.4	52.6	63.1	54.9
Average weight of young at 28 days					
(Number of cases)	(1286)	(2081)	(1939)	(1688)	(1761)
Grams ± P.E.	38.9 ± 0.08	42.4 ± 0.06	38.9 ± 0.07	40.4 ± 0.08	44.6 ± 0.11
Coefficient of variation	10.9	10.4	11.5	11.9	10.5
Length of life					
Females					
(Number of cases)	(101)	(101)	(102)	(111)	(109)
Days ± P.E.	723 ± 12	746 ± 13	818 ± 11	739 ± 14	754 ± 13
Coefficient of variation	25.3	26.8	20.8	30.0	26.8
Males					
(Number of cases)	(70)	(69)	(66)	(76)	(77)
Days ± P.E.	658 ± 12	703 ± 11	667 ± 14	689 ± 11	681 ± 10
Coefficient of variation	22.8	19.4	26.0	21.0	20.1

[1] As skim milk powder.

[2] Number of females in breeding lots.

[3] Number of females bearing young.

The findings from these experiments have been grouped in sequence according as they afford measures of 1) rate and efficiency of growth and development, 2) adult vitality, and 3) length of life.

DISCUSSION

Rate and efficiency of growth and development. From figure 1 and table 2 it will be seen that enrichment of the basal diet 16 in vitamin A (by adding butterfat) did not increase the rate of growth as measured by gain in weight during the fifth to eighth weeks (inclusive) of the rat's age, or the efficiency of growth as measured in grams of gain per 1000 Calories or per gram of protein consumed, or the rate of development as indicated by the average age of females at birth of their first young when the sexes grew up together. On the other hand, enrichment of the original diet in calcium (with or without the added butterfat) did increase the rate and efficiency of growth and expedited development as reflected in earlier reproduction. When the diet was enriched in riboflavin (and in lesser degree in protein) as well as in calcium, there was a further well-marked increase in the rate of growth with sustained efficiency calculated per gram of protein consumed, and increased efficiency when calculated in terms of gain in weight per 1000 Calories of food intake; but there was no further increase in rate of development over that observed when the diet was enriched in calcium alone.

It should be noted that diet 165, the result of enriching diet 16 by addition of butterfat only, while increasing the intake of vitamin A resulted also, because of its increased fat content, in slight diminution of intakes of protein, mineral elements, and water-soluble vitamins. It is also worthy of note that the addition of butterfat to diet 16, while not increasing the rate of early growth did, result in slightly larger adult size as may be seen from figure 1. Also the final adult weights on the diet enriched in both calcium and butterfat (diet 166) were very slightly above those on the diet enriched in calcium alone (diet 162). On diet 167, which had essentially the same

calcium content as diet 162 but was about one-fourth richer in protein (with a higher proportion of milk protein) and nearly twice as rich in riboflavin, a much more rapid early growth resulted, and a somewhat larger adult size in both sexes. During the early period in which growth was distinctly more rapid on diet 167 there was a somewhat greater food consumption per gram of body weight on this than on the other diets; but after the early period the food consumed per unit of body weight at any given age was practically the same on all five of these diets. The detailed records of food consumption are therefore omitted to save space.

Adult vitality and length of life. Throughout their lives these experimental animals were individually examined and weighed at least weekly. Records of systematic observations of appearance, alertness, condition of skin and coat, and 'feel' of muscular tone have thus been available to aid the interpretation of the numerical data.

Throughout youth and early adult life the appearance of the animals on diet 167 as judged by the condition of the coats, and firmness of body was superior to all others. Animals on diet 165 had a softer hair, and they, as well as those on diets 162 and 166, maintained their youthful appearance longer than those on diet 16.

It was also noticeable that the animals on diet 167 (richer in riboflavin and also of somewhat higher protein content) did not maintain their superiority of physical appearance in their old age. Senility set in at about the same age, and appeared to progress somewhat more rapidly with them than with the animals on diets enriched only with calcium and/or butterfat.

The numerical data used as criteria of adult vitality are 1) the time of maturity of the females as shown by their age at the birth of their first young, 2) their ability to bear and rear young, and 3) the weight of the young at 28 days of age. The outstanding features of these data are summarized in table 2. These later effects of the different enrichments of diet did not in all respects parallel those observed in early

growth. Both calcium and skim milk additions (diets 162, 166 and 167) led to significantly earlier maturity and to higher body weights of offspring; while the females receiving additional calcium or butterfat (diets 162 and 165) showed greater duration of breeding period, larger numbers of young born and reared, and larger percentage of young reared.

While on diet 16 nearly 11% of the females were sterile and 20% of those bearing young failed to rear any, these percentages were reduced on all of the other diets. In other words, the proportion of families enabled to leave descendants was increased when the basal diet was enriched in its calcium content, its butterfat content, or in its content of skim milk solids which was here chiefly significant as markedly increasing the riboflavin as well as the calcium intake, while the protein intake was simultaneously increased in much smaller proportion.

From the data of table 2 it is apparent that, as in the case of the other criteria of nutritional well-being, the increased length of life previously obtained by doubling the amount of milk in the diet (comparisons of diets A and B) was not due to any single factor in the milk, but to a combination of factors. In every case of dietary enrichment here studied, the life span has been somewhat increased; but it is not apparent why males appear to benefit more by the enrichment of the diet in its calcium content and females by the addition of butterfat. A possible explanation of the latter fact may be that the females on diet 165 were on the average about 20 days older when their first young were born, and while they reared as many as were reared on the other diets, the average weight of the young was less, so that the females on this diet might be regarded as having encountered less drain upon their vitality during the reproductive period.

If the increase in length of life on each enriched diet, over the average for the same sex on diet 16, were to be interpreted as if it stood alone, some would and others would not appear to be statistically significant; but, as already explained, each of these differences is a part of the difference hitherto

measured between the effects of our diets A and B upon nutritional well-being and resulting health and length of life. Thus it now becomes apparent that the enrichments in calcium, in vitamin A, and in riboflavin (with its accompanying minor increase in protein) have all contributed to the far-reaching improvement in nutritional well-being which resulted from the better quantitative adjustment in the proportions of natural foods in an already adequate diet.

McCay, Crowell and Maynard ('35) have recorded an experiment in which the slowing of growth apparently increased the average length of life, while in both earlier and later experiments with our diets A and B (Sherman and Campbell, '24, '35 a) we have found the latter to result both in more rapid growth and in longer life. The findings of the two laboratories may be entirely consistent. The starting point of the Cornell experiments was a diet extremely rich in protein and vitamins, whereas ours was an inexpensive dietary composed mainly of wheat and with only slightly above minimal adequate proportions of calcium, of protein and of vitamins A and G (riboflavin). Obviously there is no inconsistency in the view that limitation of intake of a very rich dietary such as theirs, and the enrichment of a dietary such as our diet A, may both be advantageous to the nutritional well-being of the life cycle. There remains a large area of investigation as to what types of diet are advantageous, respectively, to growth, to longevity, and to both. All the experiments in the present paper deal with differences of the order of magnitude of those existing between our diets A and B. Table 2 permits the comparison, for males and females separately, of the data on each of four different enrichments with the data on the basal diet 16 (diet A). Where early growth was slowed by the addition of butterfat to the basal diet, both sexes lived longer; but also when early growth was more rapid on a diet enriched in calcium, in calcium and butter, or in calcium, riboflavin and protein, both sexes here also lived longer on each of the three enriched diets which had expedited their growth. It is consistent both with our

other findings and with those of McCay, Crowell and Maynard that of the diets here studied the one which was richest in protein (and riboflavin) and which induced the most rapid growth in both sexes gave intermediate lengths of life both with females and with males. As pointed out in a previous paper (Sherman and Campbell, '35 b) it may be that the number of young produced and suckled by the females should be considered in connection with their longevity records. We hope to throw further light upon these problems through experiments now in progress.

SUMMARY AND CONCLUSIONS

The complete life cycles were studied in from 66 to 77 males and from 101 to 111 females on each of the five diets here compared. Several objective criteria of nutritional well-being as reflected in growth and development, adult vitality, and length of life, were measured quantitatively and the data submitted to critical statistical examination.

Several chemical factors are thus found to participate in the enhancement of nutritional well-being and increase in the length of life, which resulted from the improvement of an already adequate diet by increasing the proportion of milk in the food supply. The enrichments here studied were in each case of the same degree as the difference between our diets A and B.

Increased intake of calcium, either alone or with butterfat, resulted in definitely more rapid growth; while enrichment with butterfat alone seemed to result in slower growth but larger adult size than on the original diet A (diet 16).

The enrichment of the basal diet in butterfat alone had no measurable effect upon the average age at which the females bore their first young, while each of the other dietary enrichments here studied led to an earlier maturity in this respect.

Butterfat and calcium, either alone or together, resulted in longer life and an extension of the period of adult vitality as measured by the length of time during which the females were capable of bearing young. The addition of butterfat

alone did not increase the size of the young (measured by weight at the age of 28 days, taken as a conventional 'end of infancy' in this rat colony); whereas this was increased by addition of calcium either alone or with butterfat.

With both males and females, addition of butterfat alone was followed by slower growth and longer life; while in the other six of the eight comparisons, i.e., with both males and females on enrichment of diet 1) in calcium, 2) in calcium and butterfat, and 3) in calcium, riboflavin and protein, there was increase both in the rate of growth and in the length of life.

When one-fifth of the wheat of the basal diet was replaced by an equal weight of skim milk powder, i.e., when along with the increase of calcium there was a corresponding increase of riboflavin and a relatively minor increase of protein, growth was significantly more rapid than on any of the other diets; development was expedited and the life cycle extended in essentially the same degree as by enrichment in calcium alone; and larger young resulted than upon any of the other diets studied.

Thus it appears that the three factors which were increased in greatest ratio when the already adequate diet A was modified to constitute the nutritionally better diet B, i.e., calcium, vitamin A, and riboflavin, have all contributed to the improvement in nutritional well-being and resultant health and longevity.

Further studies of the relation between rapidity of growth and length of life as influenced by different modifications of diet are in progress.

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RECOVERY OF CAROTENE AND VITAMIN A FROM BUTTER WHEN COWS WERE FED UNLIMITED QUANTITIES OF GREEN RYE [1]

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The following authors have established the facts that both the carotene and vitamin A contents of butter vary widely, and that they are largely dependent on the carotene of the feeds consumed by the cow (Baumann and Steenbock, '33; Fraps and Treichler, '32; Krauss, '30; Loy, Hilton, Wilbur and Hauge, '37; Palmer and Eckles, '14; Shrewsbury and Kraybill, '33).

Moore ('32) showed that the cow is capable of converting the carotene in her ration into vitamin A in the butter fat she produces. Early investigations (Fraps and Treichler, '32; Moore, '32) have shown that only a small fraction of the total carotene ingested appears in the milk either as carotene or vitamin A.

Meigs and Converse ('33) concluded that a ration of grain and hay or grain, hay and silage was likely to be deficient in vitamin A for liberally milking cows unless it contained large quantities of good quality legume hay. After studying various rations as sources of vitamin A for dairy cows, Fraps, Copeland and Treichler ('34) concluded that apparently pasture grasses were needed to maintain the production of butter fat high in vitamin A. Krauss ('30) reported that cows doubled the vitamin A value of their butter fat when turned on pasture.

[1] Contribution no. 110, Department of Dairy Husbandry; no. 220, Department of Chemistry; and no. 64, Department of Home Economics.

Hilton, Hauge and Wilbur ('33) found that butter with high vitamin A value could be produced in winter provided good quality alfalfa or soy bean hay was fed.

Moore ('32) reported that the carotene and vitamin A content of butter never exceeds that of normal summer butter regardless of the amount consumed in the feeds. Treichler, Grimes and Fraps ('35) fed cows on a carotene-poor, vitamin A deficient diet for 60 days and then placed them on pasture for 14 days. The vitamin A content of the butter was increased from 12 units per gram to a maximum of 50 units, while the carotene increased from 0.53 to 8.03 γ per gram of butter. The amount of carotene ingested daily while the cows were on pasture was not known.

Baumann, Steenbock, Beeson and Rupel ('34) made several quantitative studies of the carotene consumed in the feeds by different breeds of dairy cows and the carotene and vitamin A recovered in the butter fat. On a winter ration the average daily intake of carotene per cow was equivalent to 240,000 international units of vitamin A, but only 8244 units were secreted in the milk. When the hay and silage of the winter ration were replaced with freshly cut alfalfa hay to simulate pasture conditions, an average of 1,040,000 units were ingested daily per cow and only 14,000 units were recovered in the butter fat. Of the carotene ingested on the winter ration an average of 1.12% appeared in the butter fat and 0.4% on the summer ration. The vitamin A in the butter fat from the two rations represented only 1.6 and 0.7% respectively of the carotene in the rations. Most of the cows in the Wisconsin experiment were consuming about 50 pounds of green material, with an average maximum of 65 pounds, whereas it is known (Woodward, '36) that cows will consume nearly three times that amount of fresh green grass.

Although several investigators have reported the carotene and vitamin A content of butter when cows are on pasture, the above experiment is the only one known to the writers in which the daily intake of carotene was known.

In view of these facts it seemed desirable to extend these studies to include cows which were consuming fresh green feed in larger quantities and the intake of carotene known, in order to measure the recovery of carotene and vitamin A in butter under pasturage conditions.

EXPERIMENTAL

The three cows used, two Ayrshires and one Holstein, were typical pure breds and averaged about a pound of butter fat daily in production. For several months previous the cows had received the regular herd grain mixture, sorghum silage and alfalfa hay. During the experiment the cows were individually stall fed green rye only. Each cow was offered in

TABLE 1
Description and production of cows used

EAR TAG NUMBER OF COW	BREED	BODY WEIGHT	GREEN RYE CONSUMED DAILY	WATER IN RYE	DRY MATTER CONSUMED DAILY	MILK PRODUCED DAILY	FAT IN MILK	FAT PRODUCED DAILY
		pounds	*pounds*	%	*pounds*	*pounds*	%	*pounds*
239	Ayrshire	955	136.5	82.3	23.9	37.2	3.8	1.4136
240	Ayrshire	1120	143.1	82.3	25.0	22.5	4.0	0.9000
118	Holstein	1196	144.2	82.3	25.2	21.2	4.0	0.8480
Average			141.3	82.3	24.7	27.0	3.9	1.0539

two feeds daily all the rye she would consume. A sufficient supply for 1 day's feeding was cut early each morning. The rye had been pastured previously and was approximately 15 inches high. It was not as green and succulent as previously and about one-fourth of the plants had headed. The cows were fed green rye 16 days, the last 3 days being used as the experimental period.

A representative sample of the rye was prepared for analysis by cutting as finely as possible with shears immediately after collection. Carotene analysis was made according to previously described technic (Guilbert, '34; Peterson, Hughes and Freeman, '37). Butter samples for analysis were obtained during the last 3 days of the rye feeding period by saving all the milk produced daily by each cow. The milk from each

cow was separated in a small cream separator and the complete 3-day collection of cream churned by hand. The carotene content of each of the three butter samples was determined spectophotometrically on the petroleum phasic fractions of aliquots which had been hydrolyzed and separated in the usual manner (Guilbert, '34). Vitamin A was determined on the non-saponifiable residues of each of the three butter samples (Report of the sub-committee on determination of unsaponifiable matter in oils and fats, '33) and the concentration determined from the intensities of the absorption band at 328 μ, as obtained from plates exposed in a Bausch and Lomb quartz

TABLE 2

Carotene content of rye, and the carotene and vitamin A content of butter

EAR TAG NUMBER OF COW	CAROTENE CONTENT PER POUND			VITAMIN A PER GRAM (EXCLUSIVE OF CAROTENE)		
	Fat in butter	Butter	Butter fat	Butter	Butter fat	
	%	*mg.*	*mg.*	*gamma*	*gamma*	*I.U.*[1]
239	82.7	2.52	3.05	10.4	12.6	19.7
240	86.5	2.27	2.62	10.1	11.8	18.4
118	84.1	2.35	2.79	9.7	11.5	17.9
Average	84.4	2.38	2.82	10.1	12.0	18.7

[1] One gamma of vitamin A = 1.56 I.U. (Morgan, R. S., J. Edisburg and R. A. Morton. 1935. Biochem. J., vol. 29, p. 1645).

NOTE: Green rye contained 25.1 mg. carotene per pound as fed, or 142.0 mg. on a dry basis.

spectrograph equipped with a Hilger rotating disc and quartz biprism. Though the ultraviolet absorption at 328 μ has now been generally accepted to be a measure of the content of vitamin A (League of Nations Commission, '34), we are aware that the method has its limitations. It is questionable, for example, whether all substances other than vitamin A which absorb at 328 μ can readily be eliminated without any loss of vitamin A. Furthermore, Pritchard and Wilkinson ('37), studying the discrepancies between biological and physicochemical methods of vitamin A assay, have reported the existence of fractions which do not correspond with the physical and chemical criteria usually accepted for vitamin A, but are biologically very active.

DISCUSSION

The carotene ingested daily averaged 3.507 gm., equivalent to nearly 6,000,000 international units of vitamin A. This emphasizes the enormous intake of carotene when cows are on pasture. Under such conditions it is not surprising that cows which have been on low carotene diets for some time have been found to re-establish the carotene and vitamin A content of their butter to a point equal to summer butter in from 2 to 3 weeks when turned onto pasture (Treichler, Grimes and Fraps, '35). It is of interest to note that the three cows studied consumed practically the same amount of dry matter and as a result had approximately the same intake of carotene.

The butter produced by the cows had the following carotene content in international units of vitamin A per pound: no. 239—5080, no. 240—4370 and no. 118—4650, averaging 4700 units. Vitamin A content of the butter in international units for the respective cows was 8944, 8354 and 8127, averaging 8490. Although these values are lower than has been reported by some authors (Sherman, '37), they are quite similar to results obtained for butter from the same breeds at the Wisconsin station (Baumann, Steenbock, Beeson and Rupel, '34), when cows were stall fed 50 pounds of green feed. They are likewise in reasonable harmony with values obtained on butter produced from pasturage at the Texas station (Treichler, Grimes and Fraps, '35), especially considering the fact that in the latter work the total vitamin A value was determined by biological analysis and the vitamin per se obtained by subtracting the value of the carotene from the total.

Thus by comparison it may be concluded that the feeding of carotene at the rate of approximately 1,000,000 international units daily resulted in butter typical of grass produced butter; but when the daily intake of carotene was increased to approximately 6,000,000 units, which is probably more typical of pasturage conditions, the carotene and vitamin A content of the butter remained relatively unchanged. This is in harmony with Moore's ('32) statement that the carotene and vitamin A

content of butter cannot be increased beyond the normal of typical summer butter.

Great waste in the utilization of carotene must, therefore, be expected under pasturage conditions. In this experiment, where an average of 5,846,000 international units were fed as carotene, an average of only 0.086% of carotene was recovered daily in the butter, while 0.154% was recovered as vitamin A, or a total of 0.24% in terms of international units. When about one-sixth as much carotene was fed, Baumann et al. ('34) recovered in the butter an average of 1.12% as carotene and 0.4% as vitamin A, making a total of 1.52%, or approximately six times the percentage recovered in the case where six times as much was fed. On winter rations

TABLE 3

Daily carotene intake of cows and daily recovery of carotene and vitamin A in butter (in international units)

EAR TAG NUMBER OF COW	DAILY INTAKE OF CAROTENE	DAILY RECOVERY			PER CENT VITAMIN A POTENCY DUE TO CAROTENE	PER CENT RECOVERED		
		Carotene	Vitamin A	Total		Carotene	Vitamin A	Total
239	5,656,000	7186	12,643	19,829	36.2	0.127	0.224	0.351
240	5,917,000	3930	7,518	11,448	34.3	0.066	0.127	0.193
118	5,964,000	3943	6,892	10,835	36.4	0.066	0.116	0.182
Average	5,846,000	5020	9,014	14,034	35.5	0.086	0.154	0.240

(240,000 units daily) they recovered in the butter 1.6% as carotene and 0.7% as vitamin A. Not only is a relatively small proportion of the carotene ingested recovered in the butter fat but apparently the greater the amount consumed the smaller the percentage recovered, at least, when generous amounts are provided.

It is interesting to note the close agreement of the results obtained on the three cows in regard to "per cent of vitamin A value in the butter due to carotene" (table 3). The average figure of 35.5% is in close accord with an average of about 32% computed for the same breeds from the results reported on their summer ration by Baumann, Steenbock, Beeson and Rupel ('24). In fact, even though the vitamin A was determined by a different method, i.e., biological assay, the report

of Treichler, Grimes and Fraps ('35) is in harmony with these results for cows on pasture. They reported 23, 30, 33 and 41 for four Jersey cows, a breed which is known to produce butter relatively higher in carotene in proportion to vitamin A than are Holsteins and Ayrshires.

It would seem, therefore, that when excessive amounts of carotene are fed daily there is tendency for the ratio of carotene to vitamin A per se in the butter to become rather constant at about 1 part of carotene to 2 parts of vitamin A, at least with Holsteins and Ayrshires (high vitamin A and low carotene breeds).

The fact that the three cows varied considerably in daily production might indicate that the ratio of carotene to vitamin A in the butter is not influenced by plane of production. Examination of the data reported by Baumann et al. ('34), and by Treichler et al. ('35) indicates that this constant ratio probably would not prevail at lower levels of carotene intake. In fact, the latter work might indicate that as the carotene in the ration is reduced, the percentage of the total vitamin A value of the butter as carotene is reduced.

SUMMARY

To simulate pasturage conditions, three cows, two Ayrshires and one Holstein, were fed all the fresh green rye they would consume for 16 days. During the last 3 days the carotene ingested daily, and the carotene and vitamin A output in the milk were computed. Daily intake of carotene averaged 3.507 gm., equivalent to nearly 6,000,000 international units of vitamin A. The butter produced had an average carotene content per pound of 4700 international units, and 8490 units as vitamin A, making a total vitamin A value of 13,190 international units. Of the carotene ingested daily only an average of 0.086% was recovered daily as carotene in the butter and only an average of 0.154% as vitamin A, or a total of 0.24% in terms of international units. The percentage of the total vitamin A value in the butter due to carotene was quite uniform for the three cows, averaging 35.5%. The data seem to

indicate that when excessive amounts of carotene are fed, the ratio of carotene to vitamin A in the butter becomes rather constant at about 1 to 2, at least with Holsteins and Ayrshires (high vitamin A and low carotene breeds).

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LOSSES OF VITAMIN C DURING THE COOKING OF SWISS CHARD [1]

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TWO FIGURES

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Swiss chard was chosen for study because it is an easily grown hardy vegetable with a long growing season, also because it is rich in minerals and, presumably, also in vitamin C as are spinach and other greens. The only study on the vitamin C of chard found in the literature was that carried out by Wasson ('31). This experiment was on raw chard only.

Wasson ('31) reports that 2 gm. daily of the green leafy portion (with the white rib removed) of fresh raw Swiss chard was not sufficient to prevent scurvy in guinea pigs; that even 4 gm. prevented scurvy in only one case out of six although all the animals gained in weight. She also found that 4 gm. of the white rib was not protective. Recalculated on the basis that 0.5 mg. ascorbic acid per day is required to protect a guinea pig from scurvy (Bessey and King, '33), this means that the green leafy chard contained less than 0.125 mg. of ascorbic acid per gram and the white rib considerably less than this amount. Wasson ('31) concludes that "the vitamin C potency of Swiss chard is very low." She did not name the variety of chard used or the kind of soil on which it was

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[2] Buhl Foundation Research Fellow. Publication no. 347 from the chemistry department, University of Pittsburgh.

grown. The chard was freshly cut, but Wasson reports that the animals on the green leafy portion were slow about eating it and some of them would have part of the chard left in their cages after 5 or 6 hours. This would probably decrease the amount of vitamin C present in the chard by the time it was consumed.

The purpose of this experiment was to determine not only the vitamin C content of the raw chard, but also that of the cooked chard and the cooking water at the 'done' stage and at various intermediate stages. The amounts of ascorbic acid reported are those obtained after reduction with hydrogen sulphide inasmuch as this represents the biologically active vitamin C present. The aim in cooking was to make the cooked vegetable look attractive and taste well, with the loss of nutrients a secondary consideration.

EXPERIMENTAL

Two varieties of Swiss chard, Lucullus and Fordhook, were grown on upland Ontario clay loam soil at Geneva, New York, and were cut during July. The Lucullus is dark green and is the market preference at present due to the fact that it does not wilt as readily as the lighter colored chard. Both varieties have large white midribs. Vitamin C determinations were made of the leaf with the midrib left intact, with the midrib removed, and of the stems alone. Analysis was also made of leaves which were chopped and those which were cut with scissors. The chard was gathered, washed, the clinging moisture removed by means of electric fans, the stems removed, the uncut leaves cooked and analyzed in as short a time as possible.

In cooking, a 3-quart enamel pan with an inside diameter of 7 inches was used. The pan and gas burner were placed on a balance so that the weight of the pan and its contents was obtainable at any stage. A manometer was connected between the gas supply and stove so that it was possible to regulate the amount of evaporation of the cooking water making it practically the same in successive cookings. Three hundred

and fifty grams of chard (four servings) were used with 1200 cc. of water and 1½ teaspoons of salt. The chard was dropped into the rapidly boiling water and the time counted from the beginning of cooking, as the water did not stop boiling. The tap water was boiled several minutes before the chard was added. An arbitrary stage of 'doneness' was determined in advance by several judges, testing with a fork and 'biting.' The boiling time for both varieties was 10 minutes. Due to the fact that it was impossible to secure samples of whole leaves (vitamin C was found to be unevenly distributed in the green leafy part and the white portion of the midrib) during the cooking and also to standardize the draining of the samples it was found necessary to have separate cookings for each of the intervals analyzed, namely, 2, 4, 6, 8, 10 ('done'), and 14 ('overdone') minutes. At the end of each of these cooking periods the chard was drained for 1 minute, samples of whole leaves removed and the remainder weighed immediately. Samples of the cooking water were also removed and the remainder weighed immediately. The weights of the total chard and the total cooking water were thus easily obtained.

After trying 8% acetic, 8% trichloracetic, 4% lactic, 1 N and 2 N sulphuric acids, all containing 2% metaphosphoric acid, it was decided to use 8% acetic acid for extracting as it gave as clear a solution as any of the acids and also resulted in a color which gave as little trouble as any acid in duplicating the titration end point.

The method of sampling was the same as that reported by Fenton, Tressler and King ('36). The method of extraction, of standardization of the dye, and titration were essentially those of Bessey and King ('33) but modified by using for the extraction an acid solution containing 2% metaphosphoric acid according to Mack and Tressler ('37). The 2,6 dichlorophenolindophenol dye was standardized with pure ascorbic acid. A total of 100 cc. of acid was used, 30 cc. being used in grinding the chard with sand in a mortar; the remainder was used in three rinsings. The first mixture was centrifuged and the liquid decanted into a 100-cc. volumetric flask. The rins-

ings were added, the mixture centrifuged and the liquid decanted between each addition of acid. Aliquots were titrated with the dichlorophenolindophenol dye immediately.

Since it was found that during extraction with the acetic metaphosphoric acid mixture there was considerable oxidation of the titratable ascorbic acid to the dehydro form, aliquots were taken and the reversibly oxidized ascorbic acid was reduced immediately according to the general method of Tillmans, Hirsch and Jackisch ('32) with the following modification: hydrogen sulphide was bubbled slowly through the sample for 10 minutes, the container stoppered and allowed to stand 20 minutes. The excess of hydrogen sulphide was removed with a rapid stream of carbon dioxide. The samples were then titrated with the dye, providing a measure of the total vitamin C content.

For purposes of comparison, biological assays were made on the raw and 'done' chard. The whole lot of one variety (Lucullus) for animal feeding was gathered the same day. Part of it was frozen directly with no blanching while part of it was cooked and drained, cooled in shallow pans surrounded by ice and salt and then packed, sealed, frozen, and kept in a container with dry ice.

The curative type biological assay was used, as in the earlier investigations of this series (Tressler, Mack and King, '36). Guinea pigs weighing approximately 225 to 250 gm. were placed in individual cages where they received the modified Sherman et al. basal diet ('22) (oats, bran, milk powder, irradiated yeast, salt and butterfat) and spinach until they had reached an average weight of approximately 260 gm. Animals that were subnormal in growth rate or significantly abnormal in any respect were discarded. They were then depleted of their tissue reserves of vitamin C by being kept on the basal diet only for 13 days. Weighed quantities of the test foods were fed daily during the ensuing 21 days, the quantity fed being based upon the dichlorophenolindophenol titration value after reduction with hydrogen sulphide. A feeding level of 0.5 mg. per day permits only partial recovery from mild

scurvy during the 21-day period, hence this level is suitable for optimum sensitivity.

A summary of the data is given in table 1. It is evident that the biological response of the three groups was comparable in both weight change and scurvy score. The record for the raw chard group was not quite as good as for the other two groups, however. This was probably due in part to the rapid rate of decomposition of the dehydro-ascorbic acid into its irreversible product.

TABLE 1

Biological assay of the antiscorbutic value of raw and cooked chard after storage in dry ice

TEST FOOD	WEIGHT [1] OF CHARD FED	VITAMIN LEVEL	NUMBER OF ANIMALS	AVERAGE WEIGHT AT BEGINNING OF DEPLETION	AVERAGE WEIGHT AT BEGINNING OF FEEDING PERIOD	AVERAGE WEIGHT AFTER 21 DAYS	AVERAGE GAIN	AVERAGE SCURVY SCORE
	gm.	*mg. per day*		*gm.*	*gm.*	*gm.*	*gm.*	
Vitamin solution		0.5	8	263	272	284	+ 12	3
Raw chard	1.42	0.5	6	251	291	290	— 1	4
Cooked chard	3.12	0.5	8	253	276	294	+ 18	2
Vitamin solution		1.0	4	268	284	348	+ 64	1
Negative control		0.0	4	260	298	176 [2]	−122	16

[1] Quantity fed based upon values obtained from preliminary and concurrent dichlorophenolindophenol titrations.

[2] Average weight at end of survival period.

The titration values of the raw and the cooked chard used in the biological assays, after storage in dry ice, at the beginning of and during the test period did not show significant variation beyond that inherent in sampling such products. The raw chard contained 0.35 mg. of ascorbic acid per gram, while the cooked chard contained only 0.16 mg. of ascorbic acid per gram. The reduction and titration of the vegetable extracts were always checked with a standard vitamin solution to avoid an error due to residual hydrogen sulphide or to reoxidation of the vitamin. The rate of loss in dehydro-ascorbic acid was about 50% every 3 hours when the frozen raw unblanched chard was allowed to stand exposed at room temperature.

DISCUSSION

The results of titration calculated on the wet and dry basis of the chard per gram and the results of titration of the cooking water per cubic centimeter are presented in table 2. The data in table 2 are presented graphically by figures 1 and 2. The curves so constructed show the milligrams of vitamin C per gram of the raw and cooked chard (on both wet and dry bases) and per cubic centimeter of the cooking water. The data in table 3 show the vitamin C in the total chard and in the total cooking water at various cooking intervals, also the percentage loss of vitamin C from the chard and the percentage gain to the cooking water.

The vitamin C content of the green portion of the leaves with the midrib cut out was found to be about 0.40 mg. per gram, while that of the stems was 0.08 mg. per gram (wet weight). The midrib was left in the leaves (although the stems were cut off) during the experiment as the housewife does not discard the midrib. Furthermore, it was found that upon removing the midrib the cooked leaves contained only two-thirds the amount of vitamin C per gram that they did if the midrib was left intact. This loss from the cut surface is to be expected in view of the great solubility of the vitamin in water. The presence of the midrib meant considerable variation in results, as the proportion of midrib varied in size in different leaves. The size of the leaves varied somewhat also. The extent of drainage proved to be a variable factor. There was less loss of vitamin C from raw chard when it was cut with scissors than when it was chopped with a knife due perhaps to less bruising of the tissues.

The Lucullus variety of chard contained slightly more vitamin C than did the Fordhook variety. This may be partially explained by the fact that the latter contained a larger proportion of midrib.

The average vitamin C content of the raw chard, 0.40 and 0.37 mg. per gram (wet weight) and 3.74 and 3.60 mg. per gram (dry weight), for the Lucullus and the Fordhook varieties, respectively, was greater than that found by Wasson

TABLE 2

Vitamin C losses from Swiss chard (per gram) and gain in the cooking water (per cubic centimeter)[1]

LENGTH OF COOKING PERIOD IN MINUTES[2]	LUCULLUS VARIETY			FORDHOOK VARIETY		
	Ascorbic acid			Ascorbic acid		
	Milligram per gram drained chard		Milligram per cubic centimeter cooking water	Milligram per gram drained chard		Milligram per cubic centimeter cooking water
	Wet weight[3]	Dry weight		Wet weight[4]	Dry weight	
Averages						
0 (raw)	0.43	3.8	0	0.36	3.6	0
2	0.25	2.6	0.04	0.22	2.5	0.02
4	0.21	2.4	0.05	0.19	2.3	0.03
6	0.16	1.9	0.07	0.17	2.0	0.04
8	0.15	1.8	0.08	0.14	1.8	0.05
10 (done)	0.16	1.8	0.09	0.12	1.3	0.06
Averages						
0 (raw)	0.37	3.7	0	0.37	3.6	0
10 (done)	0.16	1.8	0.07	0.14	1.6	0.06
14 (overdone)	0.14	1.2	0.10	0.10	1.0	0.08

[1] There was no interference from sampling in any of the cookings. The amounts of ascorbic acid shown in this table were those found after reduction with hydrogen sulphide.

[2] In all cases the water did not stop boiling when the chard was added.

[3] In terms of the raw vegetable these values were 0.43, 0.28, 0.24, 0.18, 0.17, 0.17, 0.37, 0.18 and 0.11, respectively.

[4] In terms of the raw vegetable these values were 0.36, 0.24, 0.22, 0.19, 0.16, 0.14, 0.37, 0.15 and 0.10, respectively.

TABLE 3

Total vitamin C in the chard and in the cooking water[1]

LENGTH OF COOKING PERIOD IN MINUTES[2]	LUCULLUS VARIETY				FORDHOOK VARIETY			
	Ascorbic acid in milligram		% of original ascorbic acid in		Ascorbic acid in milligram		% of original ascorbic acid in	
	Drained chard	Cooking water	Drained chard	Cooking water	Drained chard	Cooking water	Drained chard	Cooking water
Averages								
0 (raw)	151	0	100	0	125	0	100	0
2	100	37	66	24	85	23	68	18
4	85	58	56	38	77	33	61	26
6	64	72	42	48	66	44	53	35
8	60	77	40	51	58	51	46	41
10 (done)	59	80	39	53	50	56	40	45
Averages								
0 (raw)	134	0	100	0	130	0	100	0
10 (done)	62	66	46	49	55	57	42	44
14 (overdone)	39	83	29	62	32	73	25	56

[1] There was no interference from sampling in any of the cookings. The amounts of ascorbic acid shown in this table were those found after reduction with hydrogen sulphide.

[2] In all cases the water did not stop boiling when the chard was added.

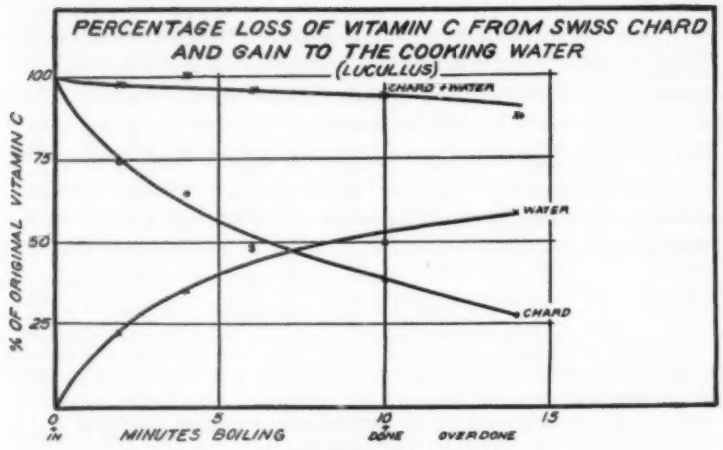

Figure 1

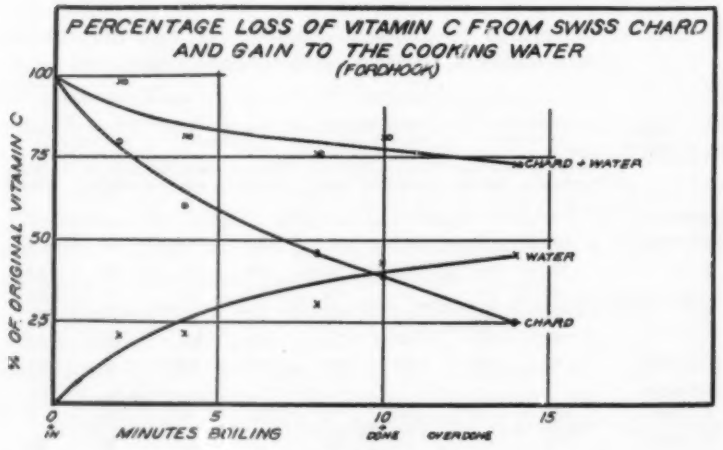

Figure 2

('31) (0.125 mg. per gram wet weight in the green leafy portion, assuming that 0.5 mg. ascorbic acid per day is required to protect a guinea pig from scurvy). In both cases the chard was freshly cut. Since the destruction of the vitamin by oxidation is very rapid in chard, Wasson's low values may be partially explained by the fact that the animals on the green portion of the leaf were always slow about eating it and some of them would have part of the chard left in their cages after 5 or 6 hours.

The fact that the reduced and the reduced plus the reversibly oxidized ascorbic acid were nearly the same after the first 2 minutes of cooking indicates that probably the so-called ascorbic acid oxidizing enzyme was inactivated during this short period. This finding agrees with that of Fenton, Tressler and King ('36) in a study of peas. Kertesz, Dearborn and Mack ('36) report that the ascorbic acid oxidase in other vegetables is inactivated by heating at 100°C. for 1 minute. The rate of loss after the first 2 minutes of cooking was decreased and most of the loss was found in the cooking water.

During a standard cooking period about half of the vitamin C was dissolved in the water. Vinokurov and co-workers ('35), Halliday and Noble ('36), Fenton, Tressler and King ('36) and Gould, Tressler and King ('36) found similarly large amounts of vitamin C lost from other vegetables to the cooking water.

The average destruction of vitamin C in the Lucullus variety at the 'done' stage was 6%, 41% being retained in the chard and 53% in the cooking water. The total destruction in the Fordhook variety at the 'done' stage was 16%, 40% being retained in the chard and 44% in the cooking water.

SUMMARY

1. The leaf portion of fresh raw Swiss chard is a good source of vitamin C (the leaf minus the stem contains about 0.38 mg. per gram).

2. The stem of fresh raw Swiss chard is a relatively poor source of vitamin C (about 0.08 mg. per gram).

3. Cooked chard contains from 0.14 to 0.18 mg. of vitamin C per gram.

4. The two varieties of Swiss chard studied contained about the same amount of vitamin C.

5. About one-half of the original vitamin C passes into the cooking water by the method of cooking used.

6. The so-called ascorbic acid oxidizing enzyme is very active in Swiss chard as indicated by the large amount of dehydro-ascorbic acid found in raw chard after extraction with acetic and metaphosphoric acid. The enzyme is inactivated during the first few minutes of cooking.

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